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# Monetarism, mayhem and research

*Dr Frank Press plans to urge on the US Administration the follies of last month's budget cut. But things are even worse elsewhere, especially in the United Kingdom.*

There is an uncanny parallel between the plight of the scientific communities in the United States and the United Kingdom. Each of them, in the past few months, has had a nasty shock as a direct consequence of the monetary policies followed by the governments in Washington and London. Each of them is faced with the nasty prospect that worthwhile projects will have to be abandoned for no better reason than that they are administratively the easiest to dispense with.

The transition from grudging contentment to despair has been the more abrupt in the United States. The second (or is it the third?) Reagan budget made public on 24 September included (apart from a modest \$2,000 million reduction of the much increased military budget) a twelve per cent cut of all discretionary public expenditure — the standard euphemism for the funds that the Administration is not by law required to spend. (The space administration is half-exempt, and required to cut by only six per cent.) The obvious difficulty is that because the reduction of public expenditure is to take effect during the financial year that began on 1 October, and before Congress has had time to weigh up the detailed plans of the federal agencies, research managers are already hard at work preparing to fire people from jobs previously thought to be secure. The immediate victims will be employees of the national laboratories. A few months from now, the chief casualties will be applicants for grants from federal agencies such as the National Science Foundation and the National Institutes of Health. The new and energetic president of the National Academy of Sciences, Dr Frank Press, has called a meeting next week in Washington at which he hopes it will be possible for the scientific community to convince government officials that the arbitrariness and the abruptness of what is now being done is a disservice not only to the scientific community but to the United States as a whole. He should not be surprised if the response is an echo of what British officials say when similarly challenged — science (or education) must shoulder an equal share of misery with other publicly supported activities, and in any case the goal of balancing the budget (or at least reducing the expected deficit) is paramount to other considerations.

In Britain, the consequences of monetarism — and of the government's animus against higher education — have been most immediately apparent in the universities. The University Grants Committee, the unwilling (but unprotesting) agent of government policy, has distributed budget reductions among British universities in such a way that none will be unharmed but that none will be literally forced out of business. The complaints, last July, that the grants committee's unspoken criteria were arbitrary is probably unfair; but the consequences of its recommendations have required arbitrary reactions of most universities. The standard procedure seems to be that a university will first look for economies that might be made by inviting those within ten years of retirement to go earlier (on the favourable terms the Inland Revenue permits), then to take advantage of the posts that will be vacated in the next three years (when the grants committee's targets for student numbers are expected to apply in full rigour) and only then to set about reducing staff numbers by compulsory redundancy. The result is that the pattern of teaching and of research is being decided not deliberately but by the personal inclinations of individual academics, the elderly and those most easily employable elsewhere. No wonder that the British research councils are alarmed that the pattern of university research in

Britain is being undermined, with too little talent (and money) spread too thinly. The next worry (to which the research councils should quickly turn their minds) is the near-certainty that in the weeks ahead, when the government's budget for 1982–83 is being hatched, the British Treasury will argue that the direct subvention of academic research should itself be cut. For if university activities have been cut back, does it not stand to reason that they need less research support?

On balance, the British research enterprise is the most seriously threatened. The direct effect of the twelve per cent edict on the budgets of the exclusively grant-making agencies (principally the National Science Foundation and the National Institutes of Health) would in ordinary circumstances be supportable. The direct effect will largely be determined by the extent to which the National Institutes of Health choose to balance the budget reductions between their in-house activities and their support of research elsewhere — a choice made all the more difficult by the continuing lack of a director to succeed Dr Donald Frederickson. In practice, however, the chief anxiety is that agencies such as the Departments of Energy and Defense, to which the support of extramural research is literally peripheral, may decide to take a disproportionate share of the economies required of them from research. Dr Press and his colleagues should not be too proud, next week, to plead for leniency from the mission-oriented agencies. But they must also, of course, do something to establish their chief point that good research enterprises cannot be turned off and on, like water from a tap. To pretend otherwise is to ignore the dependence of creative research on continuing support for productive people. Stop-go monetarism, serious enough in any enterprise, is especially damaging in enterprises in which continuity is all-important. Is the Administration prepared recklessly to squander a cohort of scientists on the thresholds of their careers for the sake of the respite their salaries and expenses will bring the Federal Research Board?

The comparable problem in Britain is less easily handled because the framework on which the budget cuts have fallen is less flexible. The universities, supported almost entirely by funds from central government, are no longer able always to function as equal partners in what is called the dual-support system — the convention that the universities provide research laboratories and the salaries of the principal investigators, while the research councils provide technical assistance, major items of equipment and small change. The most cruel feature of the arrangements decreed last July by the University Grants Committee is that some universities are left with funds with which to play their part, others have been cut back below the poverty line, but none of the parties to these implicit bargains has been told which university is in which class. The research councils are in the circumstances understandably alarmed that the universities decreed (by their recurrent budgets) to be those that cannot in future afford research will do their best to stay in the rat-race, spreading resources more thinly in the process. The research councils are understandably for more explicitly selective policies by the University Grants Committee towards the universities, but they have not yet accepted the implications for themselves — that the degree of selectivity they ask for would require that academic departments in the chosen universities should no longer be as free as they are to decide who should become graduate students.

The British problem is not merely more difficult than the



American; as things are, it is also literally insoluble. This year's cuts in university budgets will have ensured that, next year, the research councils will be seeking with less success than in recent years sparks of inspiration on which to spend their own then diminished funds; except in the most ivory of towers, demoralization has now gone too far. In the long run, British universities will survive as teaching institutions and as places at which research is carried out only if there is a greater plurality of sources of funds — among which the contribution of students towards the cost of their education must be a principal ingredient. (This last thought should publicly be suppressed, for there is a danger that the hard men at the Treasury will misread it as a proof that all support for students should be done away with and not simply as a sign that the arbitrary limits on size now imposed on individual universities are diseconomies.) Dr Press's symposium next week will be an instructive occasion. It is to be hoped that the participants can spare a thought for those even less fortunate than themselves.

## Airborne radar for all

*Selling AWACS aircraft needs diplomacy. Is the United Nations the buyer of last resort?*

When is a defensive weapon system offensive? Most simply, when it is an AWACS aircraft. This seems to be the lesson of Libya's angry reaction last week to the American decision to base two of these airborne radar aircraft in Egypt, of the earlier Israeli reaction to the United States Administration's plan to sell seven of the aircraft to Saudi Arabia — and of President Reagan's difficulty in persuading Congress that the sale should be allowed. On the face of things, a system designed to give early warning of attack would seem to be an unadulterated boon, contributing to stability and the avoidance of war. The objectors to United States plans for AWACS think otherwise. In an interesting and important way, the objections are correct.

Offensive and defensive weapons are always complementary and cannot in the last resort be sharply distinguished from each other. If one of two potential adversaries acquires a more effective system of defence, the other's offensive weapons are necessarily diminished. Thus most professional archers have been angered by the development of lightweight personal armour in the fifteenth century. The irony of the proposed sale of AWACS aircraft to Saudi Arabia is that the United States has been the chief supplier of offensive aircraft both to Saudi Arabia and to Israel, so that the transfer of the radar aircraft (already in place, but operated by the United States Air Force) would effect a retrospective devaluation of the American aircraft in service with the Israeli air force, at least in relation to Saudi Arabia. Part of President Reagan's trouble in persuading the Senate to agree to the sale — the House of Representatives has already denied him, but a veto requires unanimity of both houses — is inescapable by those who supply bows and arrows to one of a pair of potential adversaries and armour to the other. The supply of arms that neutralize each other looks foolish, escalates the level of possible combat and is a waste of resources. But the President's case would be stronger if there were some evidence that the sale would help to resolve the mounting problems of security in the Middle East.

To be fair, the Administration's case is not entirely unrespectable. Of all possible pairs of combatants in the Middle East, Saudi Arabia and Israel are the least likely. Moreover, the Saudi need of radar aircraft stems from anxiety about the stability of the Persian Gulf; the aircraft would probably spend their time looking north and east, not towards the West. But because the AWACS aircraft are capable of gathering military intelligence and because the Israelis have no assurance that information about aircraft and other military hardware within Israel would not be passed on to more probable adversaries, it is understandable that the Israeli government should have taken fright.

Yet is there nothing that might be done constructively to take the edge off this anxiety? It would be too much to ask that Saudi Arabia should forswear hostilities against Israel — in the present

climate of the Middle East, that would further inflame the opinions of other Arab states against Saudi Arabia as well as Israel. But there must be some basis for an accommodation. An agreement that Saudi Arabian AWACS aircraft should not fly over Jordan — a sizeable buffer state — would be a good start, helping to assuage Israeli susceptibilities while acknowledging Saudi Arabian sovereignty. If the Administration wants the AWACS sale to go through, it should bend its energies in that direction.

The United States should also be looking for more constructive ways of using the technology of airborne radar. These devices, the British Nimrod like the United States AWACS, are effective means of surveillance for other airborne machines, missiles as well as aircraft. Their usefulness in keeping track of what happens on the ground is less well established, although much can apparently be done. Such airborne radar systems might thus be invaluable in monitoring what happens when exhausted combatants have agreed that a United Nations peacekeeping force is preferable to the prolongation of war. As things are, airborne radar over southern Lebanon is more urgently needed than near the Persian Gulf. Is it too much to hope that the United Nations, usually the luckless peacekeeper of the last resort, will at some stage have access to this technology?

## A Daniel for the lions

*The British Government's new Science Adviser should tread carefully but also bravely. Caution will not help.*

The British government, like most of its predecessors uncertain about the quality of scientific advice it wants, this week appointed Dr Robin Nicholson as a kind of between-stairs adviser on scientific matters. Unlike legendary (and partly malevolent) people like Lord Cherwell, he will not be a confidant of the Prime Minister, but will have access to her. Unlike his immediate predecessor, Dr John Ashworth (now the unfortunate vice-chancellor of the University of Salford), he will be only partially responsible to the head of the Central Policy Review Staff. By his own account, but like earlier incumbents of similar posts, he will have to spend several months learning his way about Whitehall. In the process, like some earlier incumbents, he may be captured by the notion that government is too difficult for people whose qualifications are merely intellectual ability, technical insight and energy. Here, then, is a simple recipe for Dr Nicholson:

- Do not be patient (or too patient); this government has only two years left, and you will not be reappointed. (You have only been seconded for three years, in any case.)
- Make a constituency, even if that means spending more time talking to working scientists than to other civil servants. Even (or especially) the least revered of your predecessors have known what is happening at the bench (which is why they could often get away with murder). Giving people a sense that their opinions will be valued is good for them, while many Whitehall denizens (who travel infrequently) are mightily impressed by tales brought back from north of the River Trent.
- Write no substantial memoranda; they will be photocopied.
- Do something to help British higher education (polytechnics as well as universities) in their present plight. The colleagues with whom you will be sharing the official car-pool cannot be as indifferent as they seem to the fates of the institutions at which they were so narrowly educated.
- Do something to bring the Advisory Council on Applied Research and Development within the ordinary ambit of professional discussion; there is no *a priori* reason why it should behave as if it were another kind of think-tank.
- Do something useful; bringing Britain's defence research establishments within the scope of technical discussion is the most obvious need. Do not in the meantime worry whether the Ministry of Agriculture (to which are joined Fisheries and Food) has struck exactly the right balance between basic research and the cost of its advisory services.



# New row erupts about lab. animals

## DC indictment stirs welfare groups' protests

Washington

Public pressure for tighter laws on the use of laboratory animals has been increased by the indictment of the head of a private laboratory on the criminal charge of causing pain and suffering to monkeys. The case against Dr Edward Taub, chief investigator at the Institute of Behavioral Research in Silver Spring, Maryland, is a particular embarrassment to the National Institutes of Health (NIH), the source of Dr Taub's research contract.

Last week, animal welfare groups told a congressional subcommittee that the case demonstrates that the existing Animal Welfare Act, and its interpretation by NIH, lacks sufficient bite. Medical research organizations, keen to avoid new federal controls, countered that peer pressure and the existing guidelines were sufficient to minimize necessary suffering, and that researchers are aware that healthy animals make for better science.

The charges against Dr Taub, who had been using the monkeys for research into the recovery of stroke victims, were based on complaints made by Mr Alex Pacheco, a member of a group known as People for the Ethical Treatment of Animals. He had taken a job as a laboratory assistant at the institute, and collected data and photographs, which he used to support charges of mistreatment and unsanitary conditions.

Mr Pacheco reported his findings to the police, who have jurisdiction over the treatment of laboratory animals since Maryland is one of the few states with its own Animal Cruelty Law from which laboratories are not exempt. The police raided the laboratory early on the morning of 11 September and reported that among other things they found "monkeys in such physical and mental distress that they appeared to have bitten off their fingers and arms" — reactions which Dr Taub said were the result of removing sensation from these limbs to discover the effects on their subsequent use.

Dr Taub protests his innocence to the charges of cruelty, which if proven carry a fine of \$1,000 or 90 days in gaol, and says that Mr Pacheco's claims are based on distortion of the facts and misunderstanding of the research.

However, the case has already proved a considerable embarrassment to NIH, which have withdrawn a \$200,000 grant to the laboratory on the basis of an internal investigation, but now face accusations

that review and inspection procedures designed to prevent cruelty to laboratory animals are inadequate.

Animal welfare groups claim various inadequacies in the current law. For example, neither the Animal Welfare Law of 1970, nor amendments passed in 1976, cover rats, mice or farm animals, the most widely used groups in biomedical research.

Several bills have been introduced into Congress in the past few years to tighten up the existing law and two in particular are receiving enthusiastic support from the animal welfare community.

One would place more responsibility in the hands of institutions at which research is carried out, as well as authorize the Secretary of Health and Human Services — responsible for the NIH research programmes — to appoint an advisory committee with public representatives to keep track of the administration of animal welfare regulations. The second bill would increase support for research into alternatives to the use of live animals in research.

Some animal welfare groups want the rules to go further, for example requiring all research institutions using laboratory animals to establish animal welfare committees, with public representatives, to provide comparable protection to that given to humans used in research.

These moves are being resisted by the research community. At a hearing of the science, research and technology subcommittee of the House of Representatives Science and Technology Committee, witnesses from groups such as the American Institute of Biological Sciences, the Association of American

Universities, the National Society for Medical Research and the Association of American Medical Colleges, each argued that although existing procedures could be improved, the proposed quantum increase in regulation is unnecessary.

There was even stronger criticism of a proposal that up to 50 per cent of all NIH funds now used for work on live animals should eventually be restricted to comparable research on animal substitutes.

The research community hopes that outside criticism can be contained through a process of gradual evolution. They point out that largely because of increasing costs of maintaining animal facilities, the use of live animals in research declined by 40 per cent between 1968 and 1978. They also point to recent increases in the allocation of research funds to look at animal alternatives. Last month, for example, the Cosmetic, Toilet and Fragrance Association announced that it was making a grant of \$1 million to the Johns Hopkins School of Hygiene and Public Health in Baltimore to establish a centre for developing alternatives to animals for testing the safety of commercial products.

Whether the process of gradual change supported by the industry, NIH and by most of the research community will be sufficient to hold off new legislation remains to be seen. A decade ago, evidence such as that produced by Mr Pacheco would have virtually guaranteed the imposition of stiff new restrictions on research. But today the political environment discourages more regulation or public participation in the control of research.

David Dickson

## Handler awarded Medal of Science

Washington

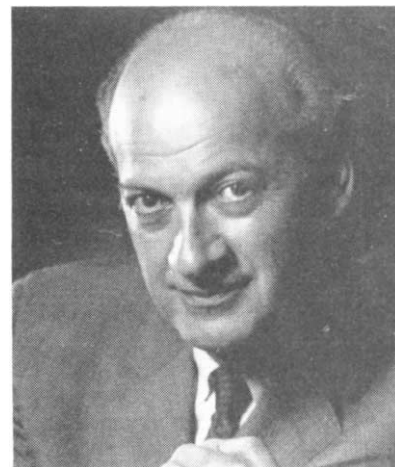
Dr Philip Handler, who retired earlier this year after twelve years as president of the US National Academy of Sciences, has been awarded the National Medal of Science, America's highest scientific award, by President Ronald Reagan for his "outstanding contribution to biochemical research" as well as for his "national leadership" in furthering the state of American science.

A statement issued last week by the White House said of Dr Handler, who was chairman of the National Science Board from 1962 to 1970, and was first elected president of the National Academy of Sciences in 1969, that "his strong and eloquent leadership of the Academy during turbulent times for science was praised widely by the scientific community on the occasion of his recent retirement".

The citation also refers to Dr Handler's research that led to a clearer

understanding of pellagra. Dr Handler, who stepped down from his academy post at the end of June, has recently been seriously ill and is receiving treatment in a hospital in Boston.

David Dickson



Philip Handler, medallist



## Yellow rain

### Waiting for data

A group of representatives of the United States government has been visiting ten European capitals during the past three weeks, presenting the evidence behind allegations by Mr Alexander Haigh, the US Secretary of State, that Russian-produced mycotoxins have been dropped on civilians in Kampuchea (*Nature*, 1 October, p.327). The representatives — who asked that they and their affiliations should not be identified — have discussed the evidence with government scientists and the press. But the evidence remains chiefly circumstantial and the release of information before the scientific evidence has been properly presented has done little to clarify whether the allegations are correct.

The mycotoxins concerned are from the tricothecene group, produced by the *Fusarium* fungi. The circumstantial evidence for their use in Laos, Kampuchea and Afghanistan consists largely of the descriptions of symptoms experienced by civilians within a few hours of the dropping of "yellow rain" from low-flying aircraft. According to the anonymous speakers at the US Embassy in London last week, the progressive symptoms of vomiting, skin blisters, haemorrhaging of mucous membranes and death were observed by journalists, doctors and refugees from these countries and, it was stated, were not consistent with "traditional" chemical warfare agents. Moreover, according to the speakers, the climatic conditions in Laos (from where the detailed evidence was drawn) are not conducive to the natural production of these toxins by fungi.

Only one sample of vegetation affected by yellow rain, taken from Laos by a person "trained in chemical warfare", has so far been analysed, and the results of that investigation — carried out by Dr C. J. Mirocha of the University of Minnesota — have not yet been accepted for publication. Dr Mirocha confirmed that three toxins, T2, nivalenol and deoxynivalenol, had been identified, and agreed with the statement that the concentrations of the last two were about twenty times the amounts observed in contaminated vegetation in other parts of the world. But he disagreed that the climate of Laos would prohibit toxin production, although he felt that other fungi would prevent *Fusarium* from producing the toxins. He felt that until his results had been published, they could not be properly discussed.

Several questions remain unanswered. None of the descriptions of observed symptoms were attributed to specific observers so that it is not possible to ascertain how objective that evidence is. The symptoms produced by mycotoxin poisoning vary greatly from species to species, according to Dr D. Paterson at the Central Veterinary Laboratory in

Weybridge, who has investigated the effects of mycotoxins on cattle. It is not obvious, therefore, that the symptoms described can definitely be attributed to mycotoxins. When asked what type of vegetation had been sampled, the government scientist replied that it was "a small bush".

Other samples are being analysed, and — as the rainy season in Laos is soon to end — it is expected that "yellow rain" may soon be falling again. But the combination of unattributed evidence, premature and incomplete discussion of scientific evidence and vested interest may have combined to defeat the purpose of the European expert tour. **Philip Campbell**

## European Community research

### Savings on defence?

#### Brussels

European Commissioner Karl-Heinz Narjes has set the cat among the pigeons by suggesting that EEC countries should co-operate on defence and military research.

This was just one of the ideas put forward in a policy paper on industrial innovation which looks at how Europe's flagging competitiveness could be improved by action at EEC level. Although only cautiously mooted by Narjes, the suggestion has led to a heated debate among the European Commissioners. Not only is cooperation on military matters excluded from the Treaty of Rome and therefore not a matter for EEC, but certain member states, notably Ireland, are already disturbed that discussions by EEC's foreign affairs ministers should include defence questions. Ireland is not a member of NATO and has neutral status.

Narjes, however, feels that the time is right to debate the advantages of sharing the costs of military research. France and the United Kingdom devote about 50 per cent of their public sector research and development budgets to military research which frequently provides valuable commercial spin-offs. France under Mitterrand is certainly more amenable to wider cooperation on defence matters and in the rest of the Community the need to keep public sector budgets pared to a minimum will surely make the idea attractive.

Another suggestion Narjes raises in the paper is to link the Community's research with the activities of the European Space Agency. The new horizons this could open up were considered in a report by European parliamentarian, André Turcat (see *Nature* 1 October, p.330).

In the main, though, the paper concentrates on more down-to-earth prospects: more innovation-orientated spending by the social and regional funds, improving access to risk capital, bringing together European companies to pool research in new technologies, and improving the incentives for investment in new technology. The European Commission has already

organized a meeting of representatives from information technologies to identify the areas in which basic and non-competitive research could take place.

A chief target of the paper is the restrictive nature of the national public supply markets. These absorb some 10 per cent of the gross domestic product and are under the immediate control of governments which, claims the commission, only favour national suppliers. These public markets are invariably in technologically rapidly evolving sectors (telecommunications, information technology, energy, transport and education) and where the successful can seldom take advantages of the large European market.

Narjes's paper will be followed by a second with more detailed suggestions that could be ready for the next European Council in London on 26 November. There the so-called May 30th mandate, which requires that the Community should move away from the heavy emphasis on agricultural spending and put more effort into industrial and social affairs, will be discussed. **Jasper Becker**

## French science budget

### More for all

Is a budget increase of — on the face of it — nearly a third for research and development, 1,800 new posts in universities and a further 630 in research agencies a vision of some glorious year of the 21st century? No, it will be France in 1982, if budget plans announced last week are put into effect.

Research and innovation are clearly a central plank in the Mitterrand government's philosophy of boom or bust; and provided inflation does not waste the rewards, French scientists seem likely to benefit greatly in the next few years.

In 1982, there should be a 20 per cent increase (less inflation) in the civil research budget. The universities will get only a 16 per cent cash increase, but many new posts, 400 of them from 1 January. Of the research agency posts, 348 will be at the prestigious Centre Nationale de la Recherche Scientifique — an increase of nearly 5 per cent on its total research staff. The university posts will be principally "assistants", junior appointments to absorb unemployed graduates of the "troisième cycle" (effectively post-docs) and rejuvenate ageing departments.

According to Jean-Pierre Chevènement, minister for research and technology, the exact allocation of the increased research budget will not be decided until after the National Colloquium on Science and Technology in Paris in mid-January. However, the division of the budget among research agencies already makes clear some rather remarkable priorities.

For example, despite the new French commitment to nuclear power (strongly championed by Chevènement), the lowest

percentage increase in research budget accrues to the Commissariat à l'Energie Atomique (CEA) (18 per cent) while the highest goes to the Commissariat à l'Energie Solaire (COMES) (50 per cent). Nevertheless, CEA has a budget of 5,200 million francs (£520 million) and COMES only 300 million francs.

Some of these increases must, however, be qualified by a French budgetary distinction between "crédits de paiement" (money actually received in 1982) and "crédits d'autorisation" (money which can be committed, as in ordering a piece of equipment, but not necessarily spent).

It is in terms of these crédits d'autorisation that there will be an increase of 29 per cent (to 25,000 million francs from 19,000 million francs). In crédits de paiement, the increase is only 15 per cent. Thus in his budget Chevènement is gambling heavily — as is all of France — on future economic success. Of what he offers to research, about half is not yet in the government's coffers. **Robert Walgate**

## UK research funding

### Cutting problems

The British Science and Engineering Research Council is alarmed that the quality of research in some outstanding science departments is being jeopardized by the reluctance of universities to be sufficiently selective in deciding where to make economies. The council's anxiety has apparently increased since the University Grants Committee informed individual universities last July of the cut in their incomes and student numbers over the next three years. Although guidance on student quotas for different subject areas was fairly specific, the council considers that advice on where to save money was too vague. It is disturbed by evidence that universities are tending to make unselective cuts.

The worry is that universities are endangering the concept of a well-found laboratory on which the dual support system for funding university research depends. The system demands that universities provide well-equipped laboratories run by tenured staff while the research councils award grants to individuals for specific research projects. The Science and Engineering Research Council intends to continue awarding grants on the merit of proposals provided that they come from laboratories with an adequate research base. But it says that if universities make cuts across the board, facilities in many departments may be so weakened that it would be unable to support their work.

That apparently has already happened on occasion in recent years. In such cases, the council has made grants conditional on the university providing the funds for basic equipment. By March 1980, the position was such that the Advisory Board for the Research Councils set up a committee to

recommend what should be done. The committee has now resumed its investigations after postponing its report when the latest cuts to the universities became known.

Sir Alec Merrison, chairman of the committee, believes that it should report by the end of the year so as to provide universities with guidance on how to reorganize while minimizing the damage to research. **Judy Redfearn**

## US engineering graduates

### Firms step in

#### Washington

Increasingly concerned at growing bottlenecks in the production of engineers by US universities and colleges, as well as the decreasing willingness of the federal government to provide financial assistance to overcome this problem, private companies are taking matters into their own hands.

Two weeks ago, member companies of the American Electronics Association, computer and data processing equipment manufacturers who have been among the hardest hit by current shortages in qualified manpower, agreed that over the next five years they would set aside two per cent of their research and development budget to assist university and college computer science and engineering departments.

This decision could produce an extra \$50 million to help support engineering education, and follows the announcement in September that the Exxon Education Foundation is to launch a five-year, \$15-million programme of assistance to engineering schools and faculty members across the country. The money will be shared among 66 colleges, including both private and state universities. Under the programme, 100 doctoral candidates will receive an average of \$50,000 over the three years of their studies. An additional \$20,000 will be provided to 100 departments of engineering, earth sciences and computer science specifically to help subsidize higher salaries for young teaching faculty members.

Although the Exxon Foundation's grant programme is said to be the largest ever undertaken by a corporate entity, it is not unique. IBM, for example, has awarded 278 graduate and predoctoral fellowships in mathematics, science and engineering in the past three years, and is making 180 grants of \$25,000 each to university departments between 1980 and 1984 — a total of \$4.5 million.

Furthermore, the private sector is so concerned that efforts to revitalize the US technological base could founder on a shortage of adequately trained engineers that Exxon and IBM have joined with six other major corporations (AT&T, du Pont, General Electric, General Motors, General Telephone and Electronics and Union Carbide) to sponsor a two-year programme in Washington under the

umbrella of the American Society for Engineering Education to consider how the federal government might help to solve the shortage of engineering college faculty.

Although pay differentials are the most widely-quoted cause for the current shortage, other factors from federal regulation of research to the obsolescence of teaching equipment — have contributed to the crisis.

There is no shortage of potential students. News of the heavy demands for engineering graduates, often being offered starting salaries of \$25,000 a year or more, has filtered quickly down into the schools, and some universities and colleges say that they are now having to limit the intake of engineering undergraduates.

According to figures being prepared by the National Science Foundation, however, private industry employers are now having "major difficulties" in filling their requirements for computer, electrical/electronic and chemical engineers. Employers had reported "some difficulties" in recruiting mechanical, industrial, petroleum and mining engineers, but said that demand and supply were in balance for aerospace engineers, while there was evidence of an oversupply of civil engineers.

Nor is it only the private sector that is worried. General Robert T. Marsh, commander of the US Air Force Systems Command is concerned that a shortage of highly qualified scientists and engineers is threatening the US defence capability.

The Reagan Administration has already advertised its concern for the general shortage of engineering graduates in both the private and military sectors. It insists that this problem should, where possible, be solved through the maximum involvement of private companies.

Some of these efforts, however, may already be beginning to backfire. Dr Ivan Bennett, dean of New York University Medical Center, has told the advisory committee to the director of the National Institutes of Health that although recent changes in the tax law make it more attractive for companies to donate second-hand research equipment to universities, some corporate leaders had told him that the value of this incentive was virtually cancelled out by the accelerated depreciation now allowed on such equipment. **David Dickson**

## Genetic engineering

### Biogen digs in

#### Zurich

Within a period of days Biogen, one of the first biotechnology companies, has opened an American counterpart to its Geneva laboratory, appointed one of last year's Nobel prizewinners as chairman of its board of directors and secured \$20 million of additional financial backing. Biogen seems bent on being among the ten



per cent of biotechnology companies that survive the next five years.

The new chairman of the Biogen board, Professor Walter Gilbert, has been closely associated with the company since its inception in 1978 both as chairman of the board of scientific directors, a role in which he will continue, and as co-chairman of the board of directors (since mid-1979). He moves to the position of chairman with the departure of the other co-chairman, Dr Robin Nicholson of Inco, to the British Cabinet Office (see p.596). Since the job of chairman is full-time, Professor Gilbert is taking a year's leave of absence from Harvard. And since he hopes to remain as chairman, he is discussing with Harvard University ways and means by which he can permanently keep both his laboratory at Harvard and his position with Biogen.

The American laboratory of Biogen Inc. opens this week on a site two blocks away from the Massachusetts Institute of Technology in Cambridge. It should be staffed with about 35 scientists by the end of the year and may well have a director within weeks. The Cambridge laboratory is destined to concentrate on the more chemical and processing sides of biotechnology, with a particular interest in bacterial methods of producing ethanol and chemical feedstocks. Meanwhile the Geneva laboratory of Biogen SA (the offspring together with Biogen Inc. of Biogen NZ, registered in Curaçao) will continue to concentrate on pharmaceuticals. The Geneva laboratory is itself due to grow rapidly, moving into new premises and doubling in size this year.

All this expansion, for a company that has not yet a single product on sale, needs just the kind of financial backing represented by the \$20 million deal closed this week with private European financial institutions (insurance companies and banks mainly in the United Kingdom). But when can the backers, which include Inco, General Metropolitan, Schering-Plough and Monsanto, expect to see some return? Professor Gilbert anticipates that Biogen's first sales should be within two years. Bacterially produced human leukocyte interferon is already in the first phase of clinical trial in the Netherlands and both a foot and mouth disease vaccine and a hepatitis B vaccine are being tested in animals. The interferon is being produced in Zurich for Schering-Plough; and Biogen has very recently concluded negotiations with the Japanese pharmaceutical company, Green Cross, for the eventual marketing of a hepatitis B vaccine.

At present Biogen relies on connections with established companies for the marketing side of its business but it plans to become in time (and in the jargon) vertically-integrated. No doubt it also plans to go public in due course but, despite speculation earlier this year, Professor Gilbert will say only that Biogen is a private company and plans to remain so for some time.

**Peter Newmark**

## Biotechnology

### UN plans centre

*New York*

An International Centre for Genetic Engineering is being planned at the United Nations. Proposed last year by UN officials, the centre would attempt to promote technology transfer between developed and developing nations in a non-commercial setting.

At a meeting in Vienna last February under the auspices of the United Nations Industrial Development Organization (UNIDO), scientists and officials from developing countries worked out a plan for a facility to extend the industrial application of genetic engineering to developing countries. The working model appears to be the International Centre for Theoretical Physics in Trieste. The centre would entertain scientists from both developed and

developing nations in order to deal with scientific problems unique to the poorer countries.

The centre would serve as an information network and as a training ground for scientists from developing countries, and might include on-site laboratories for research and development. Suggested projects already include work on vaccines for malaria and other diseases, the transfer of nitrogen-fixation genes to plants and the development of plants resistant to drought and herbicides. Agricultural specialists consulted by the United Nations foresee the creation of high technology plants capable of yielding well even with the relatively less advanced agricultural technology of the poorer nations.

There is, as yet, no indication of where the centre would be located but several countries, including Mexico and Argentina, have expressed an interest. The host country would be expected to put up money to help finance the enterprise. Other financial support would come from UNIDO funds, and UN officials say they are trying to interest several OPEC nations.

UNIDO representatives say the proposal has been well received wherever they have travelled in South America, Asia and the Middle East. But apart from the political problem of where to place such a centre, scientists researching the plan have said that some developed nations — represented in the United States by the Department of Commerce and the State Department — have been hesitant to commit themselves wholeheartedly to such a broad transfer of technology.

In addition, some Third World research directors have suggested that they would prefer that the United Nations should invest in factories rather than in building a research centre. They argue that many nations which need the fruits of recombinant DNA technology lack a nucleus of native scientists capable of taking advantage of such a facility.

The next UNIDO meeting takes place in Vienna this month, and the United States, Japan, Pakistan, India, The Netherlands, West Germany and the United Kingdom have so far agreed to attend. Final decisions about the size and location of the proposed centre are expected by January 1982.

**Michael D. Stein**

## Research in Norway

### Oiling the wheels

*Bergen*

A Norwegian government commission says that spending on the country's industrial science and technology research is grossly inadequate and that a doubling of expenditure in the next few years is needed. It is hoped that industry itself would provide most of the money, although the commission, chaired by banker Lars Uno Thulin, urges the public sector to take an initiative by spending an extra £25 million



during the next three or four years.

The mandate of the Thulin commission was to assess the effectiveness of publicly

## Plutonium deal

The United States is seeking plutonium from Britain to meet the shortages caused by the expanding US nuclear weapons programme. The US Department of Energy has approached its opposite number in Britain through the British Foreign Office with a request for plutonium reprocessed from spent reactor fuel at the Sellafield plant in Cumbria (previously called Windscale).

Negotiations are only at a preliminary stage. How much plutonium is at issue, the price, when it would be needed and the ratio of isotopes it would contain are uncertain. But, according to the British Foreign Office, negotiations concern only reactor grade plutonium for civil uses, in particular for the prototype fast breeder reactor at Clinch River, Tennessee, which the Reagan Administration hopes to persuade Congress to complete. Any agreement, says the Foreign Office, would comply with international safeguards.

The argument goes that the deal will allow the US Department of Energy to release plutonium from civil uses for nuclear weapons production. A shortage of weapons-grade plutonium by the end of the decade was forecast by the Carter Administration in its last few months of office. Hence output from reactors at Savannah River dedicated to weapons-grade plutonium production has been stepped up and another reactor will be reopened in 1984 after 13 years.

The nuclear reactor in Richland, Washington, previously dedicated to producing fuel-grade plutonium for civil use, is also being adapted to weapons-grade production. When conversion is complete in about two years' time, the stock of fuel-grade plutonium will be about 17 tons, sufficient to supply the civil programme including Clinch River until 1990. The three alternatives for ensuring supply beyond 1990 are imports, production from new equipment which could be built at Savannah River, and reprocessing spent fuel from commercial reactors. Although the Reagan Administration plans to resume reprocessing, the proposal has not yet gone to Congress.

British exports of plutonium are not unprecedented. Since 1971, Britain has exported a total of 1,280 kg for civil purposes to several countries including the United States. And it has also exported an unspecified amount to the United States under a military assistance agreement which came into force in 1959, in exchange for enriched uranium and tritium for use in Poseidon and Polaris missiles.

**Judy Redfearn**

financed technological research in an economy increasingly dominated by the North Sea oil sector but otherwise plagued by high costs and declining productivity. Industrial research is dominated by the Royal Norwegian Council for Scientific and Industrial Research (NTNF), which naturally came under the closest scrutiny.

Although much of Norway's technological research is financed directly by industry or government outside the NTNF system, each year some £80 million of public funds is channelled through NTNF to institutes wholly or partly owned by the council. In its report to the Norwegian Minister for Industry, the Thulin commission argues for NTNF to be given greater powers to decide how and where funds are to be allocated — the commission, NTNF and its constituent institutes are all critical of the recent trend for the four ministries sponsoring NTNF to earmark funds for particular projects rather than allow NTNF to decide on distribution. The commission concludes that the ministries do not have the scientific expertise required for this task.

To help NTNF to concentrate on "strategic" decisions, the commission suggests that the burden of short-term control should be transferred to the institutes themselves, by turning them into independent units funded by NTNF. This is radical surgery but NTNF, according to its managing director Gudmund Harlem, is not in principle opposed to such a move.

The council is already considering revamping its many coordinating committees in the direction of another of the commission's suggestions, namely to integrate them into one large "industry committee". This, though, is a sensitive area, since these committees are NTNF's pride and joy, on which representatives of industry, the ministries, local government, the institutes, universities and institute employees meet and provide a generally effective forum for discussing industrial research.

The most dramatic proposal in Tulin's report is a doubling of total national industrial research spending in the next few years. Norway's industrialists are taken to task for not spending enough on research and it is from the industrial sector that most of the new money should come, according to the commission, with the emphasis on applied rather than pure research.

The radical changes in the system proposed in the commission's report have, not surprisingly, met with opposition from many quarters, particularly from some of the pioneers of Norwegian industrial research in the late forties and fifties — NTNF itself was set up in 1946 and has been steadily increasing in size and influence since then. But it is difficult to believe that change will not be forced on a system which must somehow keep pace with increasingly unpleasant economic realities. Whether the new conservative government, for all its sympathy for industry, can find the means remains to be seen.

**H.M. Allen**

## Badger tuberculosis

### Bring in your dead

The British agriculture ministry is appealing to the public to notify sightings of badger carcasses which it will then test for infection with bovine tuberculosis. This latest move to increase the number of notifications, first requested in 1976, comes on the heels of the ministry's announcement that it has resumed gassing badgers suspected of passing on tuberculosis to cattle.

The gassing of infected badger setts was suspended in September 1979 pending the outcome of a review of the practice by Lord Zuckerman, president of the Zoological Society of London. The gassing began in 1975 as a means of controlling the unusually high incidence of tuberculosis in cattle in South-West England. Since then, the precise nature of the link between the disease in badgers and sporadic outbreaks in cattle has been hotly disputed. Lord Zuckerman, whose review of the largely circumstantial evidence for such a link found that gassing badgers is an effective way of minimizing outbreaks in cattle, recommended in October 1980 that gassing be resumed as soon as possible.

The ministry lost little time. Details of gassings in the last two months of 1980 are contained in a recently published report, *Bovine Tuberculosis in Badgers*, prepared by the ministry as part of its new obligation to publish annual reports on the progress of the control programme. In November and December 1980 the ministry authorized the gassing of previously untreated badger setts in 12 out of 26 areas in South-West England reporting new outbreaks of tuberculosis in cattle.

The ministry's report supports Lord Zuckerman's findings that bovine tuberculosis, which had declined in the most affected areas during the earlier gassing programme, increased last year during the ban. The number of new outbreaks, however, varied considerably between areas, the hardest hit counties being Devon, with three serious new outbreaks, and Cornwall, which had more infected cattle than at any time since 1976. Although infected badgers were found near these areas, none was found near outbreaks in Somerset and Wiltshire. As many as 24 per cent of dead badgers collected in the worst affected areas of Devon were found to be infected with tuberculosis compared with 9 per cent for the South-West as a whole.

Isolated cases of other infected mammals, in particular foxes, moles and rats, have also been found, but the ministry thinks they are unlikely to be a real threat to cattle. In the meantime, therefore, it intends to continue to monitor the incidence of tuberculosis in badgers with the public's help, and will investigate routes of transmission to cattle.

**Judy Redfearn**

# CORRESPONDENCE

## Ethics for animals

SIR — As noted in your editorial (*Nature* 17 September, p.173), human research is already controlled by ethics committees, and so we have some experience that can predict the effects of introducing their non-human counterparts.

An ethics committee that takes itself seriously is a major obstacle to a research worker. By the nature of things, its members are senior, having their own productive research days well behind them. The supplicant is called upon to justify his proposition in advance of the evidence, so the more original "look-see" type of work is discouraged. In arguing a nice point of logic, the committee is both cross-examining counsel and judge. Research workers have many possible deadlines to meet, and delay imposed by waiting for one or maybe more meetings may give the lead to other workers or cause an intending financial sponsor to take his grant elsewhere.

But, having said all that, the ethics committee is a necessary evil, and the adversarial system sharpens the scientist's mind wonderfully. Since non-human animals cannot be consulted or give consent, their "rights" must be safeguarded even more carefully than those of humans. I have never met a vivisectionist who approached his work thoughtlessly, yet standards of what is acceptable vary enormously, and if nothing else we should each be exposed to other people's opinions when planning animal experiments.

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## Survivalist hopes

SIR — Barrie Pearson (*Nature* 3 September, p.6) objects to Antony Flew's (*Nature* 16 July, p.192) "formulation of the principle of natural selection as non-random survival" on the grounds that it is an "empirically empty concept if the range of possibilities the non-random survivors were chosen from is unknown". Thus, he would have us believe that without such absolute knowledge biologists, as "creatures of reason", must accept the possibility of random survival. This argument, however, is simply another one of those "philosophical muddles" to which Dr Flew referred that continue to obscure the creationist debate.

The "range of possibilities" on which natural selection acts is ultimately dependent upon randomly occurring mutations. Many of these are deleterious, often with fatal consequences. It is not necessary to identify every possible mutation, its effects and its frequency of occurrence, therefore, in order to conclude that some allelic variants are much less likely to persist than others.

Of course, Darwin's theory of natural selection explains not only the elimination of deleterious mutations, but also the spread of favourable ones. The generality of this theory lies in the generality of certain necessary conditions. (1) Many more individuals are produced than can possibly survive; (2) among these individuals there is heritable variation in characteristics affecting reproductive success.

If Dr Pearson wishes to dispute the general applicability of natural selection he should probably begin by attacking the ubiquity of these conditions in nature. I think that it is safe to say, however, that he will not meet with much success.

ROBERT P. GENDRON

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## Gold solution

SIR — Your editorial on returning to the gold standard (*Nature* 24 September, p.246) showed an amazing degree of understanding of the topic. Gold has been a monetary standard primarily because almost all that has been mined in the past several thousand years is still around in a readily available form. Mining at full tilt can scarcely make a ripple in this huge, widely distributed bulk and the major commercial uses of gold do not destroy its availability, so that the supply is essentially independent of human agency. Your suggestion of the establishment of a short-lived isotope as an alternative monetary standard would never have occurred to me.

Your observation that gold is unnecessary is correct. All that is needed is pure reason and self-restraint and the world economy will run quite smoothly. Better yet, there will be no wars.

It is, however, not strictly correct that the gold standard would force a country to balance its yearly budget. Borrowing of funds is still permitted under a gold standard. The difference is that such debts would eventually have to be repaid rather than being inflated out of existence.

DAVID DUNTHORN

CF Systems,  
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## A good read

SIR — That science is more complex that it was fifty years ago is reflected in the fragmentation of subjects into more and more specialities, each requiring at least one journal of its own to lend an air of respectability. The hard-pressed scientist wishing to keep a tenuous grasp on the broad advance of science turns, as he always has done, to *Nature*, *Science* and the like. But does the writing in these non-specialist journals now also mirror the growing complexity of science? We have applied an objective method of testing readability to letters published in *Nature* over the past fifty years and we find a highly significant decrease between 1940 and 1950. The readability after this date appears to be stable.

The test, developed by Flesch<sup>1</sup>, uses the number of syllables, words and sentences in a chosen passage to calculate the average word length (wl) and sentence length (sl). These are

then used to compute the reading ease of the passage (RE) from the equation:

$$RE = 206.835 - 0.846wl - 1.015sl$$

Flesch produced a classification of RE which is summarized in Table 1. We have chosen every twentieth letter published in *Nature* from 1930, at ten-year intervals, to 1980 and calculated RE for the first hundred words in each. The first hundred were chosen in the belief that here the authors should be writing most clearly, attempting to explain the purpose of their work and its significance to the general reader. Choosing the beginning rather than any other part also reduced the chance of encountering complex formulae and equations which do not readily lend themselves to RE calculations. Our results are shown in Table 2.

It can be seen that there is a sharp drop in RE when 1950 is compared with 1940. The Student *t*-test shows this to be highly significant ( $P < 0.01$ ). The RE changed little over the period 1950–80. Thus, our subjective impression that *Nature* was much easier to understand during the 1960s than today does not appear to be true, at least as measured by reading ease. The significant drop in RE between 1940 and 1950 was accompanied by an increase in the number of letters published in *Nature* but, interestingly, no similar reduction took place in 1960 when the number of letters published was greater than ever before.

**Table 2** Reading ease (RE) of letters published in *Nature* over half a century

| Year | No. of letters | RE (s.e.)  |
|------|----------------|------------|
| 1930 | 8              | 32.1 (6.3) |
| 1940 | 10             | 29.1 (2.6) |
| 1950 | 29             | 16.5 (2.4) |
| 1960 | 76             | 18.0 (1.5) |
| 1970 | 59             | 15.0 (1.6) |
| 1980 | 41             | 12.8 (2.1) |

It has been suggested<sup>2,3</sup> that, for a paper to be impressive, it must be almost or, even better, completely unintelligible. For example it has been demonstrated<sup>4</sup> that, in the field of management studies, a journal's prestige increased as its readability decreased. Although we are at present unable to test this idea rigorously for science journals we have used *Current Contents* to identify a dozen letters published in *Nature* since 1950 which have been cited more than one hundred times. The mean RE of these letters was 25.0 with a standard error of 4.2 and perhaps the most celebrated of these, on the helical structure of DNA by Watson and Crick<sup>5</sup>, had an RE of 51.9. These findings suggest at least that letters appearing in *Nature* need not necessarily be cloaked in obscure and difficult language to be accepted for publication or to make a significant impact on the scientific world.

● The RE of this letter is 40.0.

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M. R. GREGG

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1. Flesch, R. *J. appl. Psychol.* **32**, 221–233 (1948).
2. Siegfried, J. *J. J. polit. Econ.* **78**, 1378–1379 (1970).
3. Armstrong, J. *S. Chemtech* **11**, 262–264.
4. Loveland, J. *Acad. Manag. J.* **16**, 522–524 (1973).
5. Watson, J.D. & Crick, F.H.C. *Nature*, **171**, 737–738 (1953).

**Table 1** Flesch classification of reading ease (RE)

| RE     | Style            | Typical example |
|--------|------------------|-----------------|
| 0–30   | Very difficult   | Scientific      |
| 30–50  | Difficult        | Academic        |
| 50–60  | Fairly difficult | Quality         |
| 60–70  | Standard         | Digest          |
| 70–80  | Fairly easy      | Slick fiction   |
| 80–90  | Easy             | Pulp fiction    |
| 90–100 | Very easy        | Comics          |



## NEWS AND VIEWS

## Amazonian fishes and the forest

from Robert M. May

THE Amazon, together with its roughly 10,000 named tributaries, contains between one-fifth and one-quarter of the world's fresh water. The river delivers about  $7 \times 10^6 \text{ ft}^3 \text{ s}^{-1}$  into the Atlantic, which is more than four times the discharge of the second largest river (the Zaire, formerly Congo) and about 11 times that of the Mississippi. Swimming in the Amazon is the most diverse assembly of freshwater fishes on Earth: some 1,300 species have been recorded, but much study remains to be done, and the total number of species may well be about 2,500–3,000.

In a remarkable recent study (*The Fishes And the Forest*, University of California Press, 1980), Goulding has shown that more than 50 species of these fish — including many of the largest and most commercially important — are nourished primarily by seeds and fruit from trees in the seasonal flood plains. To understand this work, some of the geological and meteorological complexities of the Amazon basin must first be appreciated.

Most of the  $2.3 \times 10^6$  square miles of the drainage area of the Amazon is a relatively flat plain, bounded to the north-east by the Guiana Shield and to the south-east by the Brazilian Shield (both of which are constructed mostly of igneous and metamorphic rocks, partly covered with sandstone layers), and to the west by the much younger Andes. Before the major upthrust of the Andes in the Miocene, the Amazon drained west into the Pacific. Throughout most of the Tertiary the Brazilian and Guiana Shields blocked eastward drainage, and a huge lake is thought to have formed; this lake drained when the Amazon at last began to flow into the Atlantic during the Pleistocene. One practical indication of this comparative flatness of the Amazon basin is that the river remains navigable all the way to the town of Iquitos (which is only 350 ft above sea level), 2,700 miles from its mouth.

The rainfall that fuels the various tributaries of the Amazonian River system displays strong seasonality, and most of the larger tributaries show patterns of rise and fall (with amplitudes of 20–50 ft) that are remarkably constant from year to year.

The reason for these oscillations is that, in tropical regions, the months of peak precipitation tend to be those of 'high Sun', when the Sun is directly overhead at noon. Although the later stretches of the Amazon proper lie only a couple of degrees south of the equator, the Amazon basin is spread out over  $25^\circ$  of latitude; northern tributaries (such as the Rio Negro) tend to reach their peak flow in the months of the northern summer, while southern tributaries (such as the Madeira) tend to peak in the other half of the year. In the lower stretches of the river, these seasonal fluctuations tend to average out, but they can be very pronounced in those tributaries that receive significant flow from southern or northern parts of the basin. For example, at Manaus, just above the confluence of the Rio Negro with the main river (here called the Solimões), the yearly fluctuations are around 40 ft or more.

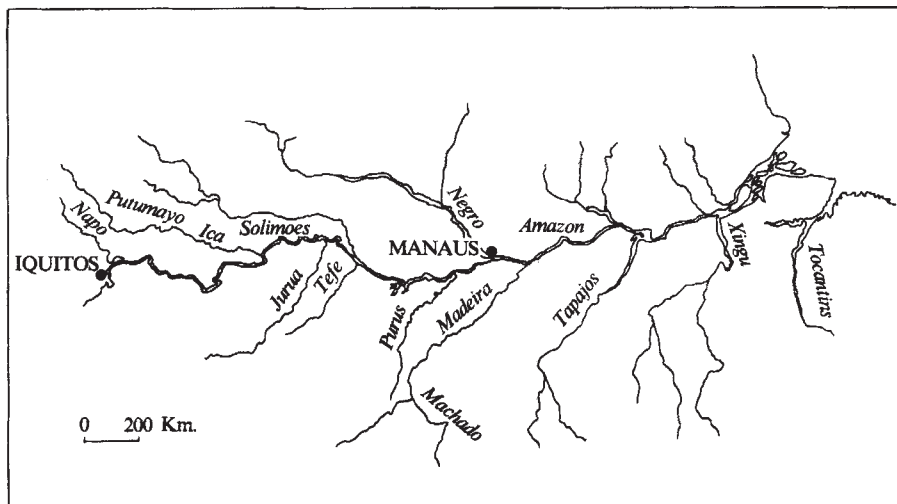
This combination of flat drainage area with pronounced seasonality in the levels of many of the major tributaries results in extensive floodplains. One estimate puts the area of the floodplain at around 40,000 square miles, or about 2 per cent of the total Amazonian basin. Helped by the favourable climate, these seasonal floodplains have produced the greatest development of flooded forests in the world.

The trees and shrubs of these flooded forest communities are highly adapted to withstand partial or total submersion for substantial fractions of the year. Goulding

writes: "Fifteen meters is the maximum depth to which I have found flooded forest trees inundated. In the deeper parts of the flooded forests, trees, shrubs, saplings, and seedlings can often be inundated for as long as eight to ten months each year; the latter three are often totally submerged during part, if not most, of this time. Only a couple of months without flooding are needed for seeds to germinate and the flood-tolerant seedlings to emerge, the latter which can then withstand the long inundations, and grow as opportunity permits each year when the floodplain is drained".

The physiological adaptations that the plants of the flooded forest must have in order to live their amphibious life are still little researched and poorly known. Goulding notes that in some cases the trees have respiratory roots (pneumatophores) which grow above the flood level, and that buttresses are also common (although these are also found on terra firma).

The ecological or biogeographical classification of Amazonian rivers remains essentially that set out by Wallace in *A Narrative of Travels on the Amazonian and Rio Negro* (Reeve, London, 1853). 'Whitewater' rivers (including the 'main stem' Solimões-Amazonas, Napo, Putumayo-Ica, Madeira, Purus and Jurua) arise in the geologically young and high Andes, and carry huge amounts of sediments that impart a turbid yellowish or cafe-au-lait colour to the river. These rivers are often characterized by extensive development of



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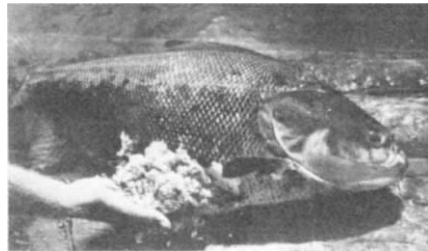
floating and attached communities of herbaceous plants, which are usually missing in 'clearwater' or 'blackwater' rivers; the transparency of 'whitewater' rivers is generally very low, from a few inches to perhaps two ft. 'Clearwater' rivers (including the Tocantins, Xingu, Tapajos and most of the large right bank tributaries of the Madeira, which includes the Machado, the main focus of Goulding's studies) arise in the geologically ancient Brazilian or Guiana Shield areas, which have been worn down by long ages of erosion. Consequently these areas release little material into the streams and rivers draining them, and these waterways have transparencies ranging from 1 to 10 ft. The poor nutrient levels generally prevent significant development of communities of aquatic herbaceous plants. Lastly, the 'blackwater' rivers (including the Rio Negro and Rio Tefe) rise in the Tertiary Lowlands; they are very nutrient poor, carry minimal amounts of suspended materials and range in colour from extremely clear to black or 'tea stained'. These rivers have been described as having chemical purity "close to that of distilled water and better than most water supplied to the public in the United States". (Incidentally, a fluid dynamicist's heart rejoices at the spectacle of the 'wedding of the waters' where the black Rio Negro and the turbid 'whitewater' Solimoes meet: differences in velocity, flow and density keep the rivers distinct, and the yellow, silt-laden Amazon and the fluid black Negro flow side by side for miles, with small yellow vortices spinning into the Negro as precursors of the eventual union).

What sustains the abundant fish life in the nutrient-poor clearwater and blackwater rivers? For many species, Goulding shows the answer is fruit and seeds from the flooded forest. His studies, begun in 1973, have involved analysis of the stomach contents of some 3,000 fishes, together with field observations, experiments and extensive interviews with fishermen and rubber collectors.

The characins are morphologically the most diversified group of Amazonian fishes. The largest family of this order is the Characidae, which includes the piranhas. It also includes the genera *Colossoma* and *Brycon*, which provide many examples of large fruit- and seed-eating fish. Preeminent are the tambaqui (*C. macropomum*) and the pirapitinga (*C. bidens*), which are among the largest scaled fish in the Amazon and are important (and delicious) food fish. The general appearance of these fish is as shown in the figure.

Altogether, Goulding has identified 9 families and 17 genera of fruit and seeds from the stomachs of large characins caught in the flooded forests of the Rio Machado. Of these, seeds without fleshy parts appear to be the most important component of the diet, whether measured by occurrence or by total volume among

the stomach contents. These large characins lurk beneath palm and rubber trees when fruit or seeds are dropping — a fact exploited by subsistence fishermen. The tambaqui appear to be particularly fond of the seeds of the rubber tree *Hevea spruceana*; of 96 such fish caught by Goulding during the flood season, 48 contained masticated seeds of this tree, and in almost all of these specimens the stomachs were full and this was the only food item (8–15 kg fish usually contained



The opened stomach of this tambaqui fish is full of masticated seeds of the rubber tree *H. spruceana*.

0.5–1 kg of seeds; see the figure). Many species of rubber trees have seed pods that explode in the hot sun and shoot the seeds distances of 30 ft or more from the tree. Goulding has painted a colourful picture of this world (*New York Times*, 15 July 1980): "In rapid succession you'd hear a 'pop' when the pods exploded, then a 'plop' when a seed hit the water and then a 'gulp' as the fish swallowed the seed".

As well illustrated in the book, many of the fish have developed large molars and specialized jaw musculatures, enabling them to crack hard foods such as Brazil nuts. Pirapitinga and tambaqui are able to crush the very hard *Astrocaryum* nut, and Goulding found one 6.3 kg fish containing just under 1 kg of the masticated fruits of this palm.

Most of the fruit- and seed-eating characins appear to build up large quantities of fat during the flood season and to fast during the dry season when the water recedes from their source. For these vegetarian fish species, the average 'fullness' of the stomachs that were analysed ranged between 72 and 93 per cent during the high-water seasons, and only 0–3 per cent had empty stomachs during this time. During the low-water period, when the fish were restricted to river channels or lakes, average 'fullness' dropped and empty stomachs were common (the fraction that were empty ranged from 25 to 75 per cent for the various species in the study). Some species turn to other food sources; for example, *C. bidens* feeds heavily on leaves during the low-water period.

Although much less abundant than the characins (especially in the medium- and large-size categories), catfishes are also represented by a diverse array of fruit- and seed eaters. As elsewhere, the Amazonian catfishes are benthic, whereas most fruits and seeds that fall into the water float, at

least initially. Thus the catfishes may be at a disadvantage compared with the characins which can intercept such food at the surface or as it sinks. Moreover, the small, villiform teeth of most catfishes are not able to masticate seeds. In partial compensation, the catfishes appear to be more active at night. All this having been acknowledged, Goulding nevertheless found fruit and seeds to be a significant part of the diet of several species of catfishes.

Overall, Goulding has shown that fish species of the same genus tend to exploit the same general kinds of fruits and/or seeds. Thus, among the characins, *Colossoma* take mostly large fruits and seeds in the deeper parts of the flooded forest; *Brycon* exploit many of the same species as *Colossoma*, but favour the shallower areas; *Mylossoma* eat mostly medium-sized seeds, as do some species of the piranhas *Serrasalmus*; *Myloes* heavily attack the seed crops of a riverside euphorb; and *Triportheus* are fond of smaller fleshy fruits. The large catfishes, *Megalodoras irwini* and *Lithodoras dorsalis*, searched out the large fruits of *Licania longipetala* in both of the flooding seasons studied.

While it is clear that the flooded forests have affected the evolution of the fruit- and seed-eating fishes, the influence of the fishes on the trees is less clear. Are the fish purely predators, or are they partly mutualistic dispersers of seeds? In the Rio Machado, Goulding found that fishes exploited a total of at least 40 species of fruits and seeds (including 38 genera and 21 families), of which 16 species were always destroyed by ingestion, while another 16 were always swallowed whole. Seeds from this second group were recovered from the lower intestines of fish, and they germinated in experimental plots. Seeds of the remaining eight species gave equivocal results. In short, seed dispersal may be helped by the fish in roughly half the cases studied. Goulding cautiously concludes: "Over long periods of time water is probably just as effective a dispersal agent for increasing the absolute distribution of a plant species in the Amazon as are fishes. Within the area of distribution, however, fishes may be the main dispersal agents that maintain the relative abundance of the species".

Goulding's study goes beyond an explanation of how fishes prosper in the clearwater and blackwater rivers of Amazonia, with their low nutrient levels. It adds a fascinating new category to the expanding list of examples of interdependence among trees and animals. It also raises worries about the future of Amazonian fisheries: the floodplains are among the more accessible parts of roadless Amazonia, and the flooded forests are increasingly being cleared to make way for rice paddies, jute fields and water buffalo and cattle ranches; fewer trees will mean fewer fishes. □



# Peregrinations: the international mobility of scientists and engineers

from Dorothy S. Zinberg

DURING the week of June 22nd some fifty scientists, engineers, government officials and research directors along with a few stray social scientists and humanists, met outside Lisbon in an opulent 18th century palace. Replete with its own chapel, sculptured gardens, fountains and lavishly-tiled rooms, the palace provided a very non-laboratory-like environment in which representatives from seventeen countries (primarily Western Europe, the United Kingdom, the United States and one witty senior science official from Japan) exchanged baleful assessments of the current state of international scientific exchanges, employment opportunities for young scientists and the future of international collaborative efforts in science and engineering research.

The meeting, the first formal international gathering on the state of international science mobility, had been preceded by almost a decade of informal meetings of high-level science and engineering officials, random studies of international travel patterns and abortive attempts to bring the subject to the attention of the scientific and engineering community. Perhaps the Reagan budget and similar signs of deep cuts in European countries provided the necessary impetus for a formal meeting, sponsored by the European Science Foundation (ESF) the National Research Council/National Academy of Sciences (NRC/NAS) in the United States and the North Atlantic Treaty Organization (NATO) Science Committee.

In the early 1970s, the Board for the International Exchange of Scientists at NAS (merged into the Commission for International Relations and later linked with the Commission on Human Resources to form a new Committee on International Human Resources which in turn organized the workshop), became concerned that all was not well with international travel related to scientific research. Although specialized meetings such as the Gordon conferences and international congresses in Europe were thriving, the once vigorous trading of younger scientists between the United States with Western Europe and Great Britain appeared to be losing momentum.

Long considered a vital aspect of scientific training for the best and the brightest, travel abroad to laboratories at the forefront of their fields had produced great science. Exuberant biographical accounts of the odysseys of physicists and molecular biologists — people such as Rabi, Szilard and Watson — have dramatized the scientific benefits of collaboration and competition among members of inter-

national networks of scientists. Is this changing? the workshop asked. Are fewer young scientists repeating the feats of their elders? Have the telephone and telex as well as rapid publication methods replaced the need to travel or are J.D. Bernal's words from 50 years ago still valid: "In spite of the best system of publication that can be devised, physical and mental techniques can generally be best transmitted by direct experience"?

As a member of the Board for the International Exchange of Scientists, I have interviewed several scores of scientists in Europe and the United States, the majority of whom thought that on average older scientists travelled where and when they wanted. It was the younger scientists who were not being brought into the international science networks. In most instances the opinions were based on changes observed in a particular laboratory or impressions from colleagues at professional meetings.

These impressions are supported by an analysis of data<sup>1</sup> from an annual study of PhD recipients in the United States which demonstrated that the numbers of first-year postdoctorals in science going abroad to Europe had indeed peaked in 1971 and continued to decline until 1979. However, first year postdoctorals are only a small percentage of postdoctorals carrying out research abroad; the majority travel during their third to fifth postdoctoral years. The data were, therefore inconclusive<sup>2</sup>.

A second study by the National Science Foundation (NSF)<sup>3</sup> revealed that a drop in foreign fellowship awards for a more comprehensive group — university faculty members — had already occurred by 1970. For example, between the early sixties and 1970, the numbers of American scientists going to Germany with NSF support had dropped by 77 per cent, to France 89 per cent and to the UK 72 per cent. Again, however, the findings were inconclusive; the numbers are small and the NSF is only a minority source of foreign fellowship funding.

In short, data to validate people's perceptions about the downturn in US research-related travel abroad were either not available, too expensive to retrieve (and those who dutifully wrote evaluation reports for NSF postdoctoral awards spent abroad were dismayed to learn that they had been filed, unanalysed, in the basement of the State Department) or, like immigration records, too crude.

At the workshop, the Royal Society

produced figures demonstrating the reality of the suspicion that there has been a dramatic reduction in the numbers of Americans going abroad<sup>4</sup>. For example, at Imperial College the number of US and Canadian senior visiting scientists spending at least three weeks at the college had dropped from 32 in 1965–66 to 12 in 1980–81. These figures also revealed how successful the ESF had been in stimulating intra-European exchanges; over the same period, the numbers of visitors had risen from 3 to 22.

The ESF director, J. Goormaghtigh, was less sanguine about the upsurge in numbers of intra-European exchanges. Like the majority of the participants, he reported that all was not well. The quality of scientific research was likely to be impaired by the ageing of scientists, the shortage of positions for young scientists and the consequent risk of there being a lack of highly qualified young people in the near future. He noted a growing tendency towards immobility based not on economic but psychological and institutional problems. Young people in large countries were suffering from acute job insecurity and did not like to leave their home countries for fear of losing employment opportunities. The participants wondered whether nationalism was extending into the world of science and engineering. The smaller countries on the other hand — the Netherlands, Sweden and Denmark — appeared to be keeping up their travel, science budgets, as evidenced by their participants' formidable language skills.

The workshop agreed that a primary goal should be to devise programmes that will encourage young people to travel and be protected for their next appointments. A more precise participant asked: "Do we want more mobility or better science? If you're at the centre of excellence, you don't have to travel around a lot". The participants also agreed that there should be continuing efforts to study industrial mobility as well as intersectoral mobility.

The consensus at the workshop appears to have been purchased by making the social sciences the scapegoat. When several of the participants (notably those from the Netherlands and Denmark), themselves natural scientists, objected to what they considered an arbitrary decision to ignore the social sciences and the humanities in the meetings, they provoked a virulent attack on social science from a NATO official who argued that the social scientists were not deserving of support, that their work was inconsequential and sometimes even destructive. Lost on the attackers was the irony that the very questions raised at this meeting — questions about psychology,

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culture, the impact of political decisions on the practice of science — were in fact social science questions and would in time be answered, if they could be answered, using those methods. The workshop also recommended that its findings should be given wide publicity, so that members of the scientific and engineering communities would be alerted to the threats to the well being of science if international activity declines. Those with data on the topic were asked to send it to the Committee on International Human Resources at the National Academy of Sciences in Washington, DC

where Walter Rosenblith, the chairman of the workshop, is attempting to establish a data bank to help foster United States exchanges with Western Europe and Japanese colleagues. □

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## Thermochronometry

from Martin Dodson

THE INTERPRETATION of isotopic ages would be a relatively simple matter, were it not for the ease with which many of the products of radioactive decay are lost from their host minerals, even at temperatures well below those at which the minerals were formed. Radiogenic strontium and argon may migrate into adjoining minerals or into the inter-granular fluid; fission tracks may disappear by annealing of the damaged crystal lattice: on slowly cooled samples therefore measured mineral ages are often much lower than the age of crystallization.

That this might be exploited to determine large-scale cooling history was first demonstrated by the Berne group<sup>1</sup> on Rb-Sr ages of micas from the Central Alps. High grade metamorphic rocks, metamorphosed at least 28 Myr ago (the oldest age obtained), gave biotite mica ages ranging from 6 to 25 Myr; muscovite micas, though fewer in number, were typically 6 to 8 Myr older than cogenetic biotites. The results were interpreted as dates of cooling through characteristic "blocking temperatures",  $300 \pm 50^\circ\text{C}$  for biotite, and  $500 \pm 50^\circ\text{C}$  for muscovite. Subsequently the picture has been filled in by K-Ar data for the micas (blocking temperatures  $300^\circ\text{C}$  for biotite,  $350^\circ\text{C}$  for muscovite) and  $^{238}\text{U}$  fission track ages on apatites (blocking temperature about  $120^\circ\text{C}$ ) to yield a fairly complete and self-consistent picture<sup>2</sup>.

Erosion and uplift rates, believed to be roughly in balance, were inferred in two ways. Linear increase of fission track ages with altitude of sample location was observed, and gave direct estimates in the range 0.2 to 1.1 mm yr<sup>-1</sup>; the excellent linear relationships also show that the present surface topography is much younger than the apatite ages. Similar rates are obtained from the ratio of cooling rate (blocking temperature divided by age) to

the estimated geothermal gradient. Past uplift rates appear to have been in some regions faster, in others slower, than present rates. In the Simplon area, where the fastest recent rates are observed, the anomalously high heat flow, which had previously been difficult to explain, can now be understood as a near-surface transient due to rapid erosion<sup>3</sup>. Furthermore, uplift rates deduced from the geochronometric data are consistent with those determined by geodetic measurements over the past few decades.

The concept of blocking temperature, central to such interpretations, is still highly problematic; not all geochronologists would agree that temperature is the key variable in the closure of a geochronometer, and those who do would not all agree on the blocking mechanism. However, for the fission track method there is no reason to doubt that the strong temperature sensitivity of lattice annealing rates is the controlling factor. Temperatures predicted theoretically by extrapolation from laboratory annealing data are supported by a remarkable direct measurement: data from a deep borehole at the Eielson Air Force Base, Alaska, showed a linear decrease in apatite fission track age with increasing depth, down to nearly zero at the base of the hole; a small extrapolation gave zero age at a temperature of  $105^\circ\text{C}$ , the inferred blocking temperature at this site<sup>4</sup>. The results imply a constant rate of uplift and erosion ( $\sim 0.05$  mm yr<sup>-1</sup>), deeper samples having cooled more recently through the  $105^\circ\text{C}$  isotherm. The slow cooling rate, compared with the Alps, may explain the slightly lower blocking temperature.

When we consider blocking temperatures for Rb-Sr and K-Ar systems we find few signs of a consensus. The Berne group chooses to infer blocking temperatures of micas from the mineral assemblages associated with "mixed ages", given by partly updated Hercynian micas in the zone of low-grade Alpine metamorphism. Implicitly they assume that temperatures

of opening of those micas during the Alpine thermal pulse are the same as closure temperatures of the same species during slow cooling of the high-grade rocks. (Indiscriminate use of the term "blocking temperature" tends to foster this risky assumption and perhaps, therefore, should be discouraged.) An alternative approach supposes the closure of minerals to be controlled by temperature-sensitive volume diffusion processes<sup>5</sup>. Assuming the Arrhenius law to be obeyed ( $D = D_0 e^{-E/RT}$ ) this work yielded a reasonable result for closure of  $\sim 1$  mm Alpine biotites, but failed to explain why very large ( $\sim 10$  cm) fissure biotites should yield the same age, and therefore have the same closure temperature, as biotites from the relatively fine-grained host rock. This points up one of the problems involved in studying diffusion minerals: diffusion geometry does not necessarily correspond to observed grain geometry. Other major uncertainties concern boundary conditions, which will depend *inter alia* on the presence and kind of fluid phase, the effect of other diffusing species competing for lattice defects, and the possible influence of temperature history on the defect structure of the lattice. Not surprisingly, good experimental data on diffusion are hard to come by, and the application of diffusion theory to mineral closure is decidedly not a routine matter.

Ideally, one would of course like to measure diffusion parameters on the actual samples which one is dating. Although at first sight a counsel of perfection, this approach has in fact been used by Berger and York in recent work on the Grenville Province<sup>6</sup> from which the title of this article is taken. They used the  $^{39}\text{Ar}$ - $^{40}\text{Ar}$  method, a variant of the K-Ar method in which  $^{39}\text{K}$  is converted to  $^{39}\text{Ar}$  by bombardment with fast neutrons. The key to their approach lies in the way argon is extracted from such irradiated samples by means of stepwise temperature increase. Isotopic analysis of the gas extracted at each step gives an age "spectrum" for each sample, and so provides a way of eliminating from the age calculation those components of the argon which give anomalously high or low ages. Its importance in the present context is that it also provides a complete diffusion experiment, and therefore an independent estimate of closure temperature, for every sample. By careful analysis of  $^{39}\text{Ar}$ - $^{40}\text{Ar}$  experiments on hornblendes, biotites, orthoclase and plagioclase feldspars, Berger and York were thus able to estimate a cooling curve extending over the time range 1,000 to 500

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Myr, with closure temperatures from 700°C to 200°C. This curve was used to draw important conclusions concerning the palaeomagnetism and polar wander path of Laurentia in the Upper Palaeozoic<sup>7</sup>.

It is easy to think of reasons why such an application of <sup>39</sup>Ar/<sup>40</sup>Ar data may be invalid. The glaring difference in boundary conditions, between the laboratory, where argon is extracted *in vacuo*, and the deeply buried rock, is the most obvious. The fact

remains that Berger and York have obtained plausible and self-consistent results, and have pointed the way for other workers using the <sup>39</sup>Ar/<sup>40</sup>Ar method to make similar analyses of their data. It is, however, very important to try to relate such results to thermochronometry based on other dating methods; only when a number of completely independent chronometric systems give mutually consistent results will it be possible to accept such cooling curves as definitive. □

anomalies. Since there are large sex differences in the proportion of children showing both physical anomalies and extremes of temperament, a defect in genetic transmission may be involved: temperamental differences may thus be genetic but not heritable. Both the importance of heritable genetic differences, and the shallowness of asking whether temperamental traits are or are not genetically determined, has been demonstrated in several comparisons of mono- and dizygotic twins. For example A. M. Torgersen (Ullevål Hospital, Oslo) demonstrated not only that the nine temperamental characteristics differed in their heritability — environmental influences being of considerable importance for some and of much less significance for others — but also that their heritability may vary with age. Thus differences in temperamental characteristics may be genetically based, yet the degree of genetic influence may vary. Furthermore, as R. Plomin (University of Colorado) emphasized, the important sources of environmental variance may differ between family members, so that intrafamily variance is as great as that between families.

In any case, temperamental characteristics may be important in personality development even if they do not depend on genetic factors, and even when they show limited consistency across age. There is, for instance, considerable evidence for a relation between temperamental and other differences in children and the development of behaviour problems either concurrently or later in childhood. For instance I. Kolvin (Nuffield Psychology and Psychiatry Unit, Newcastle Upon Tyne) demonstrated an association between overt aggressiveness in adolescent boys and a particular pattern of temperamental characteristics. However, the developmental course of such problems may involve a complex interplay between the individual and his relationships with others. In a study reported by S. Wolkind (London Hospital, London), children assessed as 'Difficult' at 4 months were more likely to develop behaviour problems at 42 months. However, the incidence of behaviour problems at 42 months related also to the mothers mental state, being greater in children of depressed mothers. The evidence suggested that an infant with a difficult temperament could affect his or her mother's later mental state to a degree that might affect the course of the child's subsequent development.

Understanding of this complex interplay between the infant's temperament and relationships with family members requires detailed study. In separate studies J. Dunn

## The study of temperament

by Robert A. Hinde

THE development of adult personality depends to a considerable extent on experiences in childhood, most of which occur in the context of relationships with parents, siblings, peers and other important figures in the child's life. The nature of those relationships are influenced by, and in turn influence, the personalities of the individuals involved. To understand the bases of adult personality we must come to terms with this dialectic between individual characteristics and relationships which starts at birth and continues throughout life. That in turn requires hard-headed methods for measuring differences between individuals.

One approach has been to focus on characteristics of 'temperament' — the style of individuals' behaviour rather than what they actually do. Although the study of temperament has a history longer than that of medicine, it was not until the 1950s that A. Thomas, S. Chess and the late H. Birch, developed a nine-variable framework within which dimensions of temperament could be reliably assessed. The dimensions concern such issues as how active the individual is in a variety of situations, how readily he (or she) adapts to new situations, whether his mood is fairly even through the day or subject to wide swings, whether his basic rhythms of eating, sleeping and bowel movement are regular or irregular, and so on. Thomas, Chess and Birch showed that individual differences in such matters could be reliably identified soon after birth and assessed throughout childhood.

Thomas and Chess were present at a recent CIBA foundation conference\* at which clinicians, psychologists and geneticists attempted to assess the current status of the approach. Not surprisingly, a central issue was that of measurement. The early work had taken advantage of parents' intimate knowledge of their children's

behaviour over a wide span of time and situations and used interviews with them to obtain data on aspects of their children's behaviour relevant to the several dimensions of temperament. A number of variants, employing interviews, questionnaires, a variety of behavioural items and diverse methods of statistical analysis have since been developed. In particular, the primary dimensions have sometimes been pooled to provide indices of the extent to which a child is 'Slow to warm up' in strange situations, and how far he is likely to prove 'Difficult' for his parents to handle. Whilst there is still need to explore new methods, the utility of the original scheme was apparent from the data presented and although parents might seem to be potentially unreliable witnesses, data obtained by independent observers of behaviour in the home showed significant patterns correlations with temperamental characteristics assessed from maternal interviews.

Individual differences in temperamental characteristics are unlikely to be useful unless they show at least some degree of consistency over time. There is little dispute that, after the age of three, considerable consistency over periods of months and even years can be demonstrated fairly readily. Indeed Thomas and Chess presented data showing some continuity in temperamental characteristics from age 4 to young adulthood. Earlier in life the evidence is more equivocal: M.O. Huttenen (Helsinki) showed significant but low correlations in temperamental characteristics between ages 6 months and 5 years.

In part because temperamental differences are present at birth, it has usually been assumed that they have a constitutional basis, being largely determined by genetic factors or by fetal or perinatal experience. Some of the most interesting discussion at the meeting concerned this issue. Data provided by R. Q. Bell (University of Virginia, Charlottesville) indicated that an aggressive/impulsive temperament factor is reliably associated with minor physical

\*The conference was organized by Ruth Porter for the Ciba Foundation, under the chairmanship of Michael Rutter (Institute of Psychiatry, London) and was held at the Ciba Foundation, London 22–24 September 1981. The Proceedings are to be published by Pitman Books, London.

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and J. Stevenson-Hinde (MRC Unit on the Development and Integration of Behaviour, Cambridge) demonstrated correlations between children's temperament and their interactions with and feelings about their mothers and other family members. Such effects also operate outside the home, for temperamental characteristics are related to interactions in the pre-school period.

Although studies of temperament in the development of personality are only just beginning, they have already been widely used by clinicians. W. Carey (University of

Pennsylvania, Philadelphia), who himself developed several widely used temperament scales, discussed how temperament assessments could assist paediatric practice both in facilitating the identification of potentially difficult children and in fostering parental understanding of difficulties. Furthermore, B. Keogh (UCLA) showed that teachers' assessments of children are affected by temperamental differences between them, indicating that an understanding of temperament is important also for educationalists.

radio-frequency interference, or in conditions of rain or sunglint. These restrictions may be eliminated in future missions by improved instrument design and data processing.

Monthly averages of SMMR SSTs are within  $0.6^{\circ}\text{C}$  of monthly averages of ship data. SMMR provided SST monthly averages more accurate than of conventional data because of the greater density of coverage. By sampling most of the world's oceans at least twice every 3 days for 3 months, the Seasat SMMR made almost a million SST measurements — a number comparable to all of the conventional SST measurements made during the past 50 years.

Wind fields from the Seasat scatterometer, have been demonstrated to be accurate to  $\pm 2\text{ ms}^{-1}$  in magnitude and  $\pm 20^{\circ}$  in direction for winds from 2 to  $24\text{ ms}^{-1}$ . However, the SASS wind measurements are obtained with as many as four wind speed directions, only one of which can be correct. Additional man-in-the-loop meteorological interpretation and processing will be required to remove the direction ambiguity. This problem can be eliminated at least 95 per cent of the time by including two additional antennas on future instruments.

Because of the lack of conventional observations, critical weather phenomena frequently pass undetected or are mislocated. The wind fields derived from scatterometer data give accurate location of weather features such as highs, lows, fronts, and tropical storms and hurricanes can be located to within 25 to 50 km.

Use of Seasat scatterometer data may also improve storm prediction. In several instances scatterometer windfields identified low-pressure areas that developed into destructive storms as much as 12 to 24h before they were detected by conventional observations. In September 1978, a rapidly developing storm in the North Atlantic was responsible for the destruction of the fishing trawler *Captain Cosmo* and the loss of its crew. In the same storm the *Queen Elizabeth II* suffered considerable damage and about twenty passengers and two crew members were injured. This storm intensified by a factor of 16 in just 24 hours. Although the conventional weather forecast predicted 2 to 3-m waves, the *Queen Elizabeth II* was pounded by 10 to 12-m waves, with occasional waves up to 16 m. Subsequent analyses suggest that Seasat data could have given advance warning of the development of the storm.

Comparison of Seasat data with *in situ* observations demonstrates that, with relatively minor exceptions, pre-launch accuracy specifications have been met or exceeded. Some problems with sensors and data products have been discovered, but these have either been solved in the course of the data analysis, or could be resolved by modifications to the sensors. As a mission to test microwave surveillance of the oceans, Seasat was an outstanding success.

## Seasat: space-age oceanography

from George H. Born

SEASAT, the first satellite designed to assess the value of microwave sensors for remote sensing of the world's oceans, was launched on 27 June, 1978. The satellite operated successfully until 10 October, 1978, when a power failure brought transmission to a stop. Despite its short lifetime, Seasat acquired a wealth of data on sea-surface winds and temperatures, ocean wave heights, internal waves, atmospheric water content, sea ice, topography of the ocean surface and shape of the marine geoid. Analysis of these data has demonstrated the success of Seasat in accurately measuring, on a global scale, important oceanographic parameters from space.

Five sensors were carried on-board the satellite: (1) a radar altimeter to measure sea-surface topography, ocean wave height, and wind speed; (2) a scanning, multi-channel, microwave radiometer (SMMR) to measure sea-surface temperatures (SST), wind speed, and atmospheric water content; (3) a wind-field Seasat-A satellite scatterometer (SASS) to measure both the magnitude and direction of the wind speed; (4) a synthetic aperture radar (SAR) for imaging the ocean's surface at radio frequency to measure wave heights and wave directions, and to recognize sea-ice features; and (5) a visible and infrared radiometer (VIRR) to provide information on cloud conditions.

For the past three years, an international team of scientists and engineers has been evaluating the performance of the Seasat sensors and analysing the data they produced. At the 1981 spring meeting of the American Geophysical Union, about 50 presentations employed Seasat data and here some of the highlights of that meeting as well as other recent research involving the altimeter, SMMR, and SASS will be discussed.

Understanding the general circulation, which governs the ocean's contribution to the global heat transport, is a fundamental problem facing oceanographers and here satellite altimetry can be of great value. The radar altimeter on Seasat measured the

distance to the ocean's surface with a precision of 5–7 cm (compared with the 1–2 m on Skylab and 30–40 cm on GEOS-3). With appropriate corrections the elevation of the ocean's surface relative to the geoid can be computed. After removing tidal effects, this topography is a manifestation of the surface pressure field from which the absolute value of the surface geostrophic velocities can be computed. Knowledge of the surface pressure field, combined with conventional in-water measurements of conductivity and temperature as a function of pressure, allows calculation of velocity profiles with depth.

Inaccuracies in our knowledge of the marine geoid currently prevent the determination of mean (time-averaged) circulation on a global basis. However, the time-variable component of ocean circulation on a global basis. However, the time-variable component of ocean circulation is being studied successfully by using Seasat altimeter data. Surface topography variations, as defined by differenced altimeter data from Seasat orbits with repeated ground tracks, depict mesoscale variability in ocean circulation patterns. Also, the combination of ocean circulation of ocean circulation measurements and wind fields such as those from the SASS will allow hypotheses of the linkages between the ocean circulation and the wind stress curl to be tested.

Synoptic SST maps have been obtained using data taken by the Seasat SMMR. With certain restrictions, temperatures from these maps have been proved accurate to  $\pm 1^{\circ}\text{C}$  over a range of 10 to  $30^{\circ}\text{C}$  when compared with ship, buoy, and bathythermograph data. At present, this accuracy is obtained by excluding data when continental boundaries are within 600 km of the instrument swath, or when the 6.6 GHz channel is contaminated by

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# Pressure demagnetization of rocks

from A.E. Mussett

IN THE EARLY DAYS of palaeomagnetism a major problem was whether rocks can retain their magnetization unaffected over millions of years. It was suggested that one of the many factors that might affect the magnetization is stress, and Graham<sup>1</sup> argued that, because of magnetostriction, igneous rocks might be affected by cooling stresses, and sediments by the loading and unloading of overburden. He went on to show in the laboratory<sup>2</sup> that uniaxial stress, equal to the weight of a column of rock half a kilometre high, changed the intensity of magnetization of most rocks, by up to a quarter or more, with accompanying directional changes. This stimulated research in a number of laboratories in the late 1950s, with the conclusion that though stress could affect magnetization it affected only the softer components and is roughly equivalent to applying an alternating field. With the development of routine 'cleaning' techniques it ceased to be a bogey and interest in the effects of stress largely faded, with the exception of its possible use as a harbinger of earthquakes.

The early work was concerned chiefly with the direction of magnetization, vital to such problems as the reality of continental drift, but in recent years interest has extended to intensity as well. Pearce and Karson<sup>3</sup> have conducted some experiments that indicate that pressure may well play an important role in situations of high pressure but low temperature, as may be found in subduction zones, meteorite impacts and the interiors of small, cool planetary bodies. They have used much higher pressures than did the earlier experimenters (up to 20 kbar, equivalent to about 60 km depth) and hydrostatic pressure, which is more realistic than uniaxial stress for great depths, but only investigated the effects of stress applied in near-zero fields. Broadly, their results confirm the earlier conclusions but with rather more pronounced effects. For instance, coercivities affected exceeded 0.05 T (500 oe) for some rocks and sometimes over half the natural remanence was removed. Such large effects are surprising with hydrostatic pressure because, for magnetostriction to operate, there needs to be a change of shape rather than size. Some of the effect may be due to the relation of the grains to the matrix in which they are embedded: if there is a difference in the elastic properties of grains and matrix an external hydrostatic pressure on the rock will generate non-hydrostatic

forces on the grains, unless the grains have a high degree of symmetry. Indeed, Pearce and Karson confirmed this by showing that samples in jackets, which prevented fluid flowing into cracks and pores and so tended to produce true hydrostatic pressure on the grains, showed larger demagnetization than unjacketed samples. But this cannot be the whole explanation for magnetite powder suspended in oil still showed a large effect. The authors offer, as a partial explanation, the known decrease of both the magnetostrictive constant and the anisotropy with increasing hydrostatic pressure, and they point out, therefore, that, their high hydrostatic pressure would enhance the effect of any residual uniaxial stress, or changes of magnetization by any process, for that matter.

Pearce and Karson suggest that pressure magnetization may help to explain the absence of magnetic anomalies near oceanic trenches, and limit the depth to which magnetization can occur in a cool planetary interior. They also suggest it may play a part in remagnetization by meteorite impacts. Another question that comes to

mind is what effect it may have on determinations of palaeointensities. Shaw's method<sup>4</sup> may have an advantage here over Thellier's method<sup>5</sup> since it usually entails demagnetizing samples to quite large alternating fields.

The ideas of Pearce and Karson are plausible; now they need to be substantiated. For instance, what happens if the Earth's field is present when pressure is applied? Further, how is pressure magnetization and demagnetization at these pressures affected by the rate of application and the duration of the pressure, since nature acts more slowly than most experimenters. Domen<sup>6</sup>, more persistent than most of us, has conducted an 11-year experiment and claims that time is an important factor. It also would be nice to have a better understanding of what is happening, though this may be asking too much, for our understanding of rock magnetism is still inadequate. Best of all would be a geological test of their ideas. □

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## Chemolithotrophic carbon dioxide fixation in tube worms — symbiotic primary production

from Don P. Kelly

THE chemolithotrophic bacteria have long been recognised as contributing to primary production through carbon dioxide fixation independent of solar energy. Sulphide-oxidising chemolithotrophs were subsequently shown to be at the base of food chains on hydrothermal vents in the Galápagos Rift and enable the growth of dense animal populations. A significant part of the carbon of Galápagos Rift mussels is derived from dissolved inorganic carbon apparently incorporated from filter feeding on such chemolithotrophs<sup>2</sup>. Recently most exciting observations have been made, showing that carbon dioxide fixation by chemolithotrophic bacteria seems to be the principal source of carbon for the vestimentiferan tubeworms such as *Riftia*<sup>3,4</sup>. These have been shown to contain chemolithotrophic bacteria in their tissues and consequently to have active CO<sub>2</sub>-fixing Calvin cycle enzymes actually within the animal body. In this issue (see p.616) Southward *et al.* have extended these studies to the smaller members of the phylum Pogonophora from various habitats. These animals lack

mouth, digestive tract and anus and consequently pose intriguing questions regarding their nutrition. The demonstration of bacterial cells in their tissues and the presence of high levels of ribulose biphosphate carboxylase indicated that the autotrophic fixation of carbon dioxide is significant in these marine worm-like animals. The <sup>13</sup>C/<sup>12</sup>C ratios in this and in other studies<sup>3</sup> support the view that much of the organisms' carbon could be derived from carbon dioxide. A picture is thus emerging of carbon nutrition among marine animals that may benefit from bacterial chemolithotrophic-energy-dependent carbon dioxide fixation not only in sulphide-rich environments but also in habitats where low levels of chemolithotrophic nutrients are available. Such symbiotic associations are clearly able to conserve to a high degree the total energy and carbon available within an ecosystem, and widen further our understanding of the role of chemolithotrophic bacteria in the environment.

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# Time to buy a squid?

from Hilton Stowell

THE past decade has witnessed the evolution of the once esoteric art of 'gross evoked potentials' into the profitable biomedical technology of 'event related brain potentials' (ERBP). The new name is both more accurate and more informative. The essential difference between traditional electroencephalography (EEG) and ERBP is this: Whereas the EEG looked at the electrical activity of the whole brain, for many seconds of time, and in much ignorance of what that particular brain was doing during that time, ERBP examines the electrical activity of cooperating networks of cortical neurones when, and only when, they are responding to specific and controllable inputs or outputs of information.

As often happens in the history of science, technological innovation has improved our appreciation of a theoretical problem. Before the invention of the 'signal averager' there was little understanding of the importance of timing for cerebral events, except perhaps among a handful of psychophysicists; it had been a sufficient achievement, in the time of Hans Berger and Lord Adrian, to make the brainwaves visible without penetrating the skull. Then the computer of average transients spotlighted time, often at the expense of space; the spatial resolution of ERBP is notoriously weak.

Now, perhaps the most significant technical advance since the birth of the 'signal averager' has occurred. This newcomer is the superconducting quantum

interference device (SQUID). Originally a geologist's instrument for detecting very weak magnetic fields, the SQUID has been applied to the analysis of human brainwaves since around the mid-nineteen-seventies in the United States. It is now accepted, both in the United States and in Europe, as a necessary adjunct to conventional electric-field recording<sup>1-3</sup>. Why is it useful?

The SQUID attacks ERBP methods at their weakest point, which is the accurate determination of cerebral sources and sinks; and the inference therefrom of cortical dipole origins for specific temporal components of the sequence of voltage polarity reversals which make up an 'evoked potential' of the brain. Its ability to do this<sup>4-6</sup> depends, in the first place, on two elementary physical properties of the magnetic field associated with every electric current: First, the magnetic field strength attenuates faster with distance from its origin than does the electric field; second, it is neither attenuated nor smeared by low-conductivity tissues such as the skull. A more trivial advantage of magnetic gradiometry is its obvious ability to do without skin-contact electrodes on the scalp, and without that pervasive nuisance of traditional electroencephalography, the hopefully 'neutral' or 'indifferent' reference electrode on an earlobe, nose-tip, or more distant part of the body which inevitably picks up the ECG also. Moreover, what might seem to be the gravest disadvantage of measuring very weak magnetic fields turns out to be yet another advantage over traditional ERBP and EEG. No magnetic shielding is needed in ordinary suburban environments<sup>5</sup>. The asymmetric

gradiometry technique is sensitive only to the magnetic field difference between its two linked, asymmetrical loops, while rejecting components of the magnetic field common to both. Of course, like its rough analogue the a.c. differential amplifier, the SQUID's common mode rejection is limited. You don't put your stimulating equipment close to the subject's head, or set up your ERBP laboratory next door to a large magnet. But the magnetic fields of neural generators close to the scalp can be quite sharply localized by a SQUID sitting 1.5cm off the scalp.

Now for the disadvantages: the SQUID double-loop must sit within a dewar of liquid helium, and this limits manoeuvrability whilst increasing cost. Worst of all, data must be signal-averaged from only one gradiometer at a time. So scalp topography must be done serially rather than in parallel from a large array of conventional EEG electrodes. The absence of messy scalp-preparation and electrode-sticking may bring a slight compensation in time-saving.

At the time of writing there may be half-a-dozen ERBP laboratories systematically using SQUIDs in the United States and perhaps two or three in Europe (one in Finland certainly). I predict that within five years it will be difficult to publish useful 'human evoked potential' data without one. □

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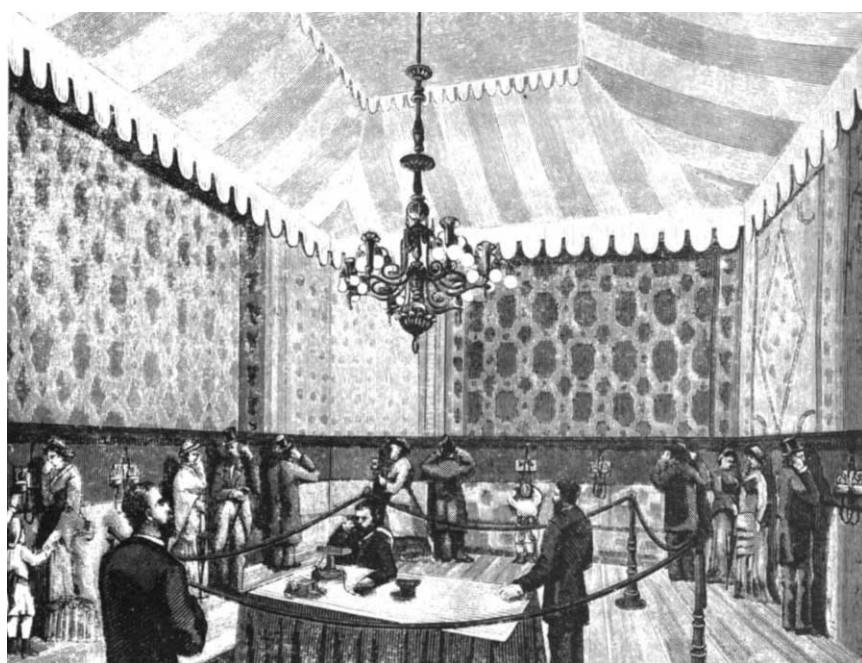
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## 100 years ago

### THE INTERNATIONAL EXHIBITION AND CONGRESS OF ELECTRICITY AT PARIS

One of the greatest successes of the Exhibition of Electricity is certainly the arrangement for the telephonic hearing of theatrical performances which has been organised by the Société Générale des Téléphones. By means of an electrical connection which has been established between the Opera, the Théâtre Français, and the Exhibition Palace, it has become possible to hear in the most complete manner the pieces played on our two principal dramatic stages. The singing of the Opera especially has a fairy-like effect, and all who have been so fortunate as to penetrate into the sanctuaries reserved for these hearings go away astonished and enchanted as if they had come from a dream of the Thousand and One Nights. The effect is in fact captivating; for the singing of the Opera is positively better heard in this way than in the Opera House itself.

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## ARTICLES

## Local structure of silicate glasses

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*Extended X-ray absorption fine structure is used to study local order in silicate glasses around sodium and silicon atoms. It is demonstrated that modifying cations like sodium have well-defined short range order that is complementary rather than incidental to the glass forming silicate network.*

THE structure of  $\text{SiO}_2$  and the silicates is dominated by the invariance of the  $\text{SiO}_4$  pyramid unit. In the crystalline polymorphs of  $\text{SiO}_2$  (ref. 1) as well as in silica itself<sup>2</sup> the Si–O bond length is 1.61 Å and the O–Si–O bond angle is 109.4°. Because in each case the connectivity of the network is complete, differences in overall structure relate to variations in the Si–O–Si bond angle and to various dihedral angle or bond rotation configurations. In the crystalline state these variations are discrete and systematic and long-range order is achieved; in the glassy state they are random and long-range order is lost. This is the basis of the continuous random network model (CRN) proposed originally by Zachariasen<sup>3</sup> and realized in later modelling studies<sup>4,5</sup>.

In silicates, modifying cations such as  $\text{Na}^+$  and  $\text{Ca}^{2+}$  break up the three-dimensional  $\text{SiO}_2$  network. In the crystalline state they promote layered and chain structures and the diversity of the minerals. In the amorphous state they lower the melt viscosity and enable stable glasses to be formed. Despite the structural upheaval the same  $\text{SiO}_4$  tetrahedral unit present in  $\text{SiO}_2$  persists in the silicates.

Although X-ray and neutron diffraction studies<sup>6</sup> of silica<sup>7</sup> and silicate glasses<sup>8</sup> have verified the tetrahedral coordination of oxygen around silicon they have been unsuccessful in deducing anything definite about the oxygen ligands surrounding modifying cations. This is due to the need to disentangle several pair correlation functions from the total diffraction intensity, a problem which in principle could be overcome using isotopic substitution or anomalous dispersion techniques. Thus the total radial distribution function has been calculated which is dominated by the well-defined and more numerous Si–O and O–O distances of the  $\text{SiO}_4$  unit; these mask any modifier–oxygen correlations. All the atomic pairings are, however, separated in the extended X-ray absorption fine structure (EXAFS) technique: Si–O distances are restricted to the Si EXAFS spectrum, Si–O and O–O distances feature in the O EXAFS spectrum<sup>9</sup> but neither Si–O nor O–O distances will make a direct contribution to the EXAFS spectrum of a modifier.

We have examined the K-edge of sodium in sodium disilicate ( $\text{Na}_2\text{Si}_2\text{O}_5$ ) and the 'universal' soda-lime-silica glass  $\text{Na}_2\text{CaSi}_5\text{O}_{12}$ ; each glass presents a very definite EXAFS spectrum establishing directly for the first time the oxygen coordination of sodium modifiers in silicate glasses. We have also measured silicon K-edge EXAFS in  $\alpha$ -quartz and silica. Results corroborate diffraction data<sup>7,8</sup> and demonstrate the reliability of EXAFS for studying multi-component solids of low atomic number.

## Spectroscopy

X-ray absorption spectra of the K-edges of silicon (6.75 Å) and sodium (11.49 Å) were measured using the soft X-ray monochromator at LURE<sup>10</sup>. This is a high vacuum instrument using synchrotron radiation from the 500-MeV storage ring ACO as a source. Low-energy radiation is filtered off from the white beam using a thin Be window ( $\sim 1 \mu\text{m}$ ) and the transmitted X rays are then dispersed using a pair of KAP crystals (Bragg spacing = 13.3 Å) arranged in the +1 –1 configuration. The intensity of

the monochromatic radiation transmitted through the specimen is measured using a single detector: an ionization chamber for the silicon K-edge and a channel plate for the sodium K-edge. The transmission spectra are normalized by a retrospective scan with the sample removed.

In these experiments the resolving power of the monochromator,  $\Delta\lambda/\lambda$ , was limited chiefly by the photon opening angle of the synchrotron radiation ( $\sim 10^{-3}$  rad) as no entrance slits were used<sup>10</sup>. At the silicon K-edge  $\Delta\lambda/\lambda$  ( $\cot \theta \Delta\theta$  where  $\theta$  is the Bragg angle) is  $4 \times 10^{-3}$  improving to  $2 \times 10^{-3}$  at the sodium K-edge. Whilst this is well matched to typical EXAFS oscillations ( $>5$  eV) it is insufficient to resolve white lines fully. In silica the white line is reported to be 2.6 eV wide<sup>11</sup>. Spectra were collected with  $\sim 1$  eV point separation and with a typical signal-to-noise ratio of  $10^3$ . Raw data for the silicon K-edge of  $\alpha$ -quartz are shown in Fig. 1a. Silicon spectra were obtained at 77 K and sodium spectra at room temperature. Further details of the instrument and soft X-ray measurements are given elsewhere<sup>10,12</sup>.

The specimen thickness  $t$  necessary to achieve an optimum signal-to-noise ratio in transmission is given by  $\mu t \sim 1$ , where  $\mu$  is the absorption coefficient.  $\mu$  increases rapidly with wavelength and for third period elements between 5 and 10 Å  $1/\mu \sim 3,000$  Å to 1  $\mu\text{m}$ . Whilst such sample thicknesses can be restrictive for crystalline specimens they pose few problems for glasses which can be readily blown into self-supporting thin films. The  $\alpha$ -quartz specimen studied here was prepared by casting a sub-micrometre powder suspended in collodion onto the surface of water<sup>12</sup>. To prevent prolonged exposure to air our films were placed under vacuum within 1 h of preparation, during which time there was no visible deterioration.

## Analysis

Normalized EXAFS spectra,  $\chi(E)$ , for the silicon and sodium K-edges are shown in Fig. 2. Data were reduced from the absorbance spectra using standard techniques (see ref. 13) and then smoothed using a cubic spline. The raw data for  $\alpha$ -quartz are presented in Fig. 1b for comparison. The most obvious common feature of all six spectra displayed in Fig. 2 is the limited range of  $\sim 400$  eV. This corresponds to an upper value of the electron momentum  $k$  of  $\sim 10 \text{ Å}^{-1}$  of which  $2k$  ( $\sim 20 \text{ Å}^{-1}$ ) will be exchanged ( $k = \sqrt{2mE}/\hbar$ , where  $E$  and  $m$  are the electron's energy and mass respectively). The short EXAFS spectra reflect the weak scattering of the light-atom environment. Backscattering amplitudes,  $f(\pi, E)$  for oxygen, sodium and silicon are shown in Fig. 3. The analogue function in X-ray diffraction is the form factor. An upper limit of  $16 \text{ Å}^{-1}$  for the X-ray momentum is typical of oxide glasses<sup>7</sup>. Clearly EXAFS and X-ray diffraction have potentially similar precision as probes for short-range order in glasses. The period of the EXAFS oscillations, however, will not exactly match those in the X-ray diffraction pattern. Although they are both dominated by the radii of near-neighbour coordination shells, EXAFS oscillations are modified by the  $k$ -dependence of the phase shifts of the photoelectron as this is diffracted by the potential of the emitting atom and its surrounding ligands.

Phase shifts for silicon, sodium and oxygen are also shown in Fig. 3.

The scattering parameters given in Fig. 3 were calculated using a Hartree-Fock muffin tin program<sup>14,15</sup>. Starting from atomic wavefunctions<sup>16</sup> this calculates the photoelectron wavefunction for a particular atomic environment and with it the phase shift. The  $\alpha$ -quartz structure was used for the silicon and oxygen parameters whilst the sodium parameters were calculated for an octahedral coordination. Madelung corrections for  $\text{Si}^{2+}$ ,  $\text{O}^-$  and  $\text{Na}^+$  were incorporated to assimilate partially covalent silicon-oxygen bonds and completely ionized sodium cations. The emitting atom phase shifts for silicon and sodium given in Fig. 2b model relaxed atoms by using atomic wavefunctions for phosphorous and magnesium respectively ( $Z+1$ )—the 1s hole is seen by the remaining electrons to increase the nuclear charge by one.

The incoherent scattering of the photoelectron is accounted for in EXAFS theory<sup>14</sup> by two damping terms: a Debye-Waller term  $\exp(-2\sigma_j^2 k^2)$  and an inelastic scattering term  $\exp(-2VR_j/k)$ . The factor  $\sigma_j^2$  is the mean square relative displacement of the distance  $R_j$  between emitting and scattering atoms. If there is no correlation between the motions of these two atoms,  $\sigma_j^2$  is just the mean square displacement due to thermal motion<sup>17-19</sup>. Correlation of the atomic motions due to long-wavelength phonons will decrease  $\sigma_j^2$  below this value<sup>15</sup>. In principle in an amorphous system static disorder will also add to  $\sigma_j^2$ . In the monatomic Debye model<sup>17</sup>,  $\sigma_j^2$  is simply related to the cutoff frequency ( $k\theta/\hbar$ ), the atomic mass and the density. Taking the bond stretching frequency for Si-O ( $1,100\text{ cm}^{-1}$  ref. 20) as a measure of the Debye frequency and the average atomic mass yields  $\sigma_j^2 = 0.0019(5)\text{ \AA}^2$ . This should be compared with the uncorrelated bond length variance obtained from pulsed neutron measurements by Misawa *et al.*<sup>21</sup> of  $0.0025\text{ \AA}^2$  for silica and silicate glasses. If correlation is included  $\sigma_j^2$  falls to  $0.0009(3)\text{ \AA}^2$ . Taking a bond stretching frequency for the weaker Na-O bond of  $240\text{ cm}^{-1}$  (ref. 20) yields at room

temperature the larger uncorrelated value of  $\sigma_j^2 = 0.037\text{ \AA}^2$ . The correlated value is  $0.035\text{ \AA}^2$ .

Inelastic losses involve the finite electron and hole lifetimes. For light atoms like silicon and sodium the latter is small. The low-energy electron, however, can excite the plasma of valence electrons and therefore has a self energy with a significant imaginary part  $V$ . Away from the energy loss peak ( $\geq 20\text{ eV}$ ) this is approximately constant. Calculations<sup>22</sup> indicate a value of  $\sim 5\text{ eV}$  for atoms as light as silicon or sodium.

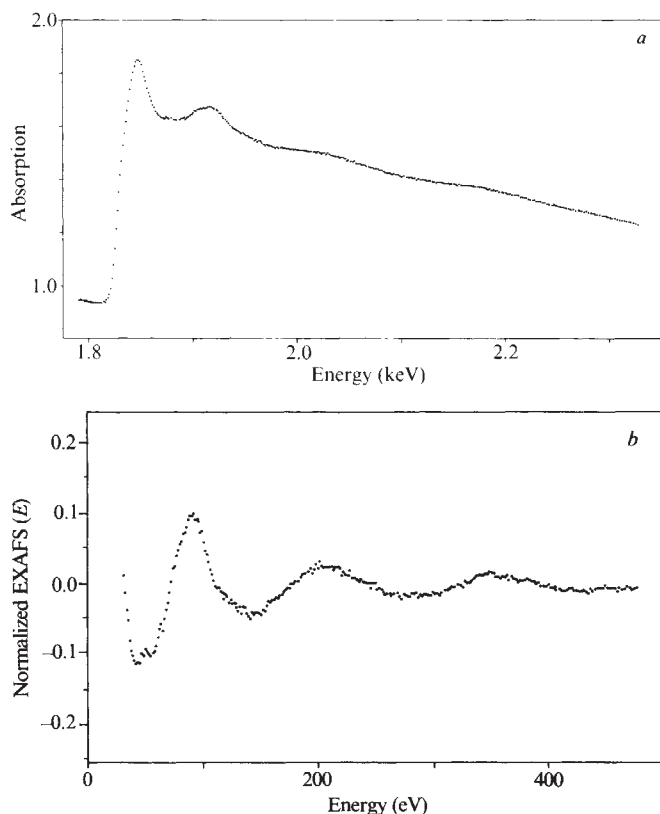
Whilst most EXAFS parameters can be calculated fairly reliably two uncertainties remain: the overall quantum efficiency of the absorption process and the energy zero of the photoelectron. In the present analysis a global shake-up-shake-off factor of 0.8 has been used. Values of 0.7–0.8 agree with many other EXAFS studies<sup>23</sup> for absorption by atoms of medium atomic number. For light atoms like Si or Na, however, we might expect a higher proportion of direct absorption events and hence a factor  $>0.8$ . But the good agreement obtained here with the known structure of  $\alpha$ -quartz suggests that errors from this source are small. The energy zero of all EXAFS spectra has been taken at 13 eV from the turning point of the Si or Na absorption edges. This parameter represents the difference between the muffin tin zero which lies near the bottom of the bound states in the valence bands and the bottom of the conduction band where the empty states start. Tight binding band-structure calculations of silica and quartz<sup>24</sup> yield a similar figure of around 13 eV. The width of the top of the valence band from photoemission studies<sup>25</sup> is  $\sim 4\text{ eV}$  wide. Adding the optical gap of 9–10 eV (ref. 26) gives an empirical total of 13–14 eV; all this indicates that the scattering factors in Fig. 3 are quite accurate.

The values for shake-up-shake-off and the energy zero have been obtained using  $\alpha$ -quartz as a model compound. This comparative approach reduces uncertainties in these and other EXAFS parameters for the glasses such as the shell radii and the coordination number. However, because there are correlations between the EXAFS parameters themselves, the final fits quoted in Table 1 are not at the statistical limit of the data. Often the strongest correlation is between the Debye-Waller factor  $\sigma_j^2$  and the coordination number  $N_j$ . However, we fit  $N_j$  at low energies where the Debye-Waller factor has little effect. We estimate that by taking  $N_1 = 4$  oxygens for  $\alpha$ -quartz  $N_1$  and  $\sigma_1^2$  for the glasses are accurate to between 10 and 20%. Outer shells will be progressively less accurate.

Atomic distributions around silicon and sodium are given in Fig. 4. These were obtained by Fourier transforming the normalized EXAFS spectra from Fig. 2 after multiplying them by  $(k/f_i(\pi)) \exp(2\sigma_j^2 k^2)$ —to remove energy-dependent amplitude factors<sup>18</sup>. This assumes the EXAFS spectrum can be represented in terms of the single scattering of plane waves<sup>14</sup>—a reasonable approximation for electron energies  $>200\text{ eV}$ . For structure closer to the absorption edge the plane wave approximation becomes progressively inaccurate. Characteristically it underestimates bond lengths and overestimates coordination numbers. This is the region where the EXAFS signal in oxide systems is strongest. Lee and Pendry<sup>14</sup> have given a more precise solution for the scattering of spherical photoelectron waves. This is used for curve fitting in Fig. 2. The Fourier transforms shown in Fig. 4 are therefore only a guide. The clusters presented in Table 1 are the result of curve fitting using the spherical wave approximation.

## Local order around silicon

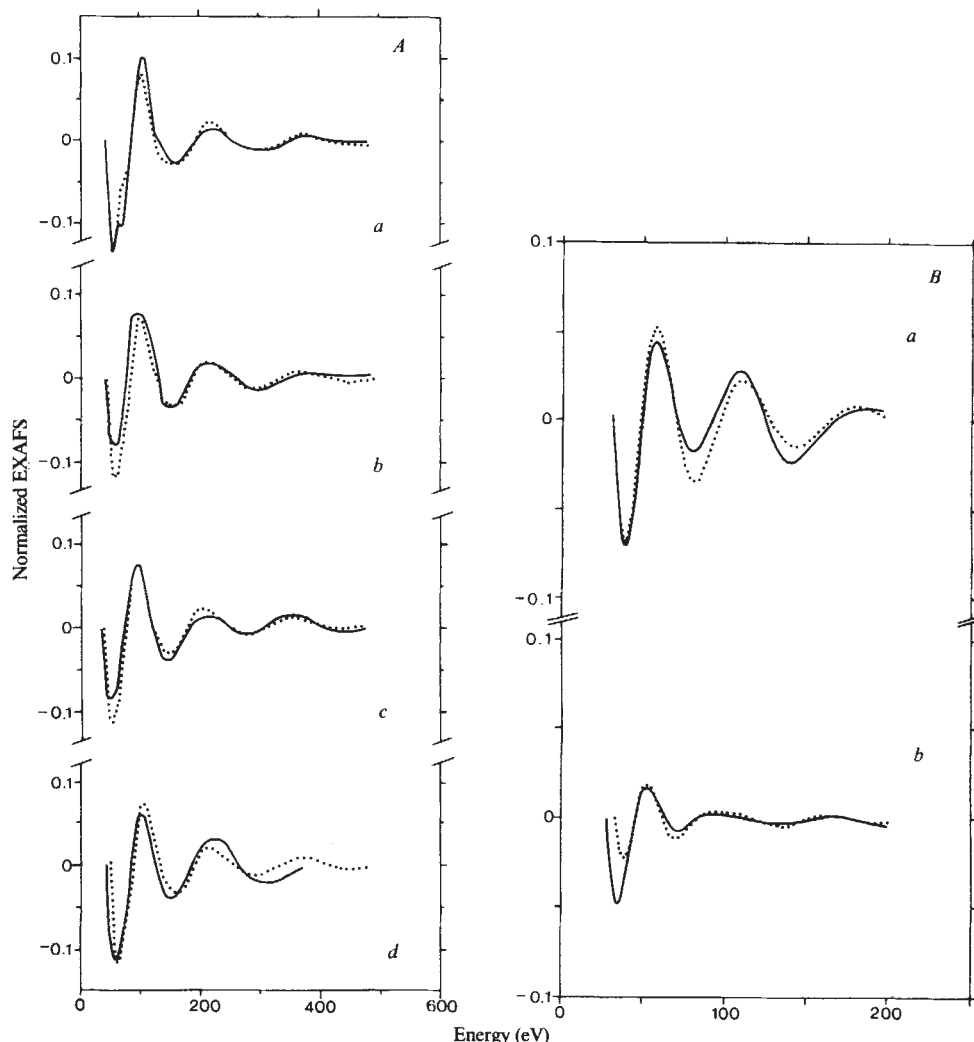
The striking feature of the Si EXAFS spectra (Fig. 2A) is their similarity.  $\chi(E)$  for each silicate glass is practically indistinguishable from that of silica and apart from some minor features all three glasses have almost the same  $\chi(E)$  as quartz. The rigid  $\text{SiO}_4$  unit is common to all four structures and this single oxygen shell dominates the EXAFS as is clearly seen in the Fourier transforms (Fig. 4A). The absence of strong structure relating to second near neighbours and beyond stems from the weakness of the Si-O-Si linkage compared with the primary



**Fig. 1** a, Raw data for the X-ray absorption of  $\alpha$ -quartz showing the silicon K-edge at  $6.75\text{ \AA}$  ( $1.84\text{ keV}$ ) and the EXAFS above the edge. b, Raw data for the normalized fine structure reduced from the absorption spectrum shown above.



**Fig. 2** A, Normalized EXAFS spectra  $\chi(E)$  above the silicon K-edge (6.75 Å) of: a,  $\alpha$ -quartz; b, silica; c, sodium disilicate glass; d, soda-lime-silica glass. Solid curves are experiment with the white line removed. Normalized EXAFS spectra  $\chi(E)$  above the sodium K-edge (11.94 Å) of a, sodium disilicate glass; b, soda-lime-silica glass. The energy zero is taken 13 eV above the tuning point of the absorption edge. Dotted curves are calculations using Lee and Pendry's spherical wave theory<sup>14</sup> with the structural parameters listed in Table 1.



Si-O bond. Bell *et al.*<sup>27</sup> for instance, estimate the bending and stretching force constants to be 70 and 490 N m<sup>-1</sup> respectively.

The results of curve fitting are given in Table 1 and theoretical and experimental  $\chi(E)$  are compared in Fig. 2A. The first shell radii for  $\alpha$ -quartz and the three glasses are 1.60 and 1.61  $\pm$  0.02 Å respectively. These agree with the X-ray diffraction values of 1.61 and 1.62  $\pm$  0.01 Å for  $\alpha$ -quartz<sup>1</sup> and silica<sup>2</sup>. The present EXAFS results also agree well with pulsed neutron measurements<sup>21</sup> for silica and sodium disilicate glass of 1.61 and 1.63  $\pm$  0.01 Å respectively. For the first shell of all four spectra  $\sigma_1^2$  equals 0.0010  $\pm$  0.0001 Å<sup>2</sup>. This agrees well with the calculated value of 0.00093 Å<sup>2</sup>, indicating substantial correlation in nearest-neighbour motion. Similar behaviour has been observed in Ge where the bonding is also strong<sup>28</sup>. The fact all three glasses share the same Debye-Waller factor as  $\alpha$ -quartz demonstrates the absence of any measurable static disorder around silicon in the glasses. In contrast crystalline silicates have considerable static disorder. Clearly local order is improved in the glassy state where the restrictions of long-range order are lifted.

In  $\alpha$ -quartz a second shell consisting of four silicons (3.06 Å) is clearly present in the Fourier transform (Fig. 4A) giving a Si-O-Si bond angle close to 144°. Also in evidence is the next shell of four oxygens (3.56 Å) (ref. 29). Curve fitting indicates these shells are responsible for the shoulders in  $\chi(E)$  at 68 eV and 148 eV (Fig. 2A). The factor  $\sigma_2^2$  for these outer shells in  $\alpha$ -quartz is 0.010  $\pm$  0.001 Å<sup>2</sup>.

Evidence for an equivalent shell of four silicons in the Fourier transforms of the three glasses is less obvious. Curve fitting, however, demonstrates that the amplitudes of the first minimum and maximum in  $\chi(E)$  are incorrectly estimated unless a second

shell is included. The best match for all three spectra involves a shell of four silicons at 3.17 Å. This shell radius is 0.1 Å longer than for  $\alpha$ -quartz and yields a larger Si-O-Si bond angle of 160°.  $\sigma_2^2$  is also greater than for  $\alpha$ -quartz, 0.021  $\pm$  0.007 Å<sup>2</sup>, implying that the static disorder in the Si-O-Si bond angle is  $\pm$  20°. Both results agree well with those of X-ray diffraction<sup>30</sup>. (The oxygen bond angle and its distortion are substantially different from GeO<sub>2</sub> glass where the respective values are 130°  $\pm$  6.5° (ref. 31).)

### Local order around sodium

Turning to the Na EXAFS in Fig. 2B it is evident first that compared with the Si EXAFS (Fig. 2A) the frequency of oscillations is higher, implying the Na-O bond is longer than the 1.61 Å Si-O bond. Second comparing the two Na EXAFS spectra themselves the amplitudes are quite different: sodium disilicate glass is the stronger, indicating a higher coordination number for the first shell. The Fourier transforms shown in Fig. 4B make these distinctions plain. Both glasses have a common first peak around 2.35 Å and this is strongest for sodium disilicate. However, for soda-lime-silica glass there is also a second shell peaking at a 'non-bonding' distance of 3.2 Å.

Curve fitting yields a first shell radius for sodium disilicate of 2.30  $\pm$  0.03 Å and a coordination number of 5  $\pm$  0.5 atoms. For soda-lime-silica the first shell is at the slightly longer distance of 2.43  $\pm$  0.03 Å and the coordination number has the lower value of 2  $\pm$  0.5 atoms. In addition, as the Fourier transform suggests (Fig. 4B), outer shells amounting to an extra four oxygens have been added (see Table 1). The first shell factors  $\sigma_1^2$  for each glass are also different: 0.056  $\pm$  0.0005 Å<sup>2</sup> for sodium disilicate and 0.014  $\pm$  0.002 Å<sup>2</sup> for soda-lime-silica. Comparing these to 0.035 Å<sup>2</sup> calculated from the Na-O stretching frequency, it is

**Table 1** Curve fitting parameters used to obtain theoretical spectra shown in Figs 1 and 2 (dotted curves)

| Sample                                                | K-edge | $N_1$ | $r_1$<br>(Å) | $\sigma_1$<br>( $10^{-2}$ Å <sup>2</sup> ) | $N_2$ | $r_2$<br>(Å) | $\sigma_2$<br>( $10^{-2}$ Å <sup>2</sup> ) | $N_3$ | $r_3$<br>(Å) | $\sigma_3$<br>( $10^{-2}$ Å <sup>2</sup> ) |
|-------------------------------------------------------|--------|-------|--------------|--------------------------------------------|-------|--------------|--------------------------------------------|-------|--------------|--------------------------------------------|
| $\alpha$ SiO <sub>2</sub>                             | Si     | 4 0   | 1.6(0)       | 0.1(0)                                     | 4 Si  | 3.0(6)       | 1.0(0)                                     | 4 0   | 3.5(6)       | 1.0(0)                                     |
| SiO <sub>2</sub> (g)                                  | Si     | 4 0   | 1.6(1)       | 0.1(0)                                     | 4 Si  | 3.1(7)       | 2.1(1)                                     |       |              |                                            |
| Na <sub>2</sub> Si <sub>2</sub> O <sub>5</sub> (g)    | Si     | 4 0   | 1.6(1)       | 0.1(0)                                     | 4 Si  | 3.1(7)       | 2.1(1)                                     |       |              |                                            |
|                                                       | Na     | 5 0   | 2.3(0)       | 0.5(6)                                     |       |              |                                            |       |              |                                            |
| Na <sub>2</sub> CaSi <sub>5</sub> O <sub>12</sub> (g) | Si     | 4 0   | 1.6(1)       | 0.1(0)                                     | 4 Si  | 3.1(7)       | 2.1(1)                                     |       |              |                                            |
|                                                       | Na     | 2 0   | 2.4(3)       | 1.4(4)                                     | 1 0   | 2.8(6)       | 2.8(8)                                     | 3 0   | 3.3(3)       | 2.6(6)                                     |

Atom type and number are given by  $N_i$ , the shell radius by  $r_i$  and the Debye–Waller factor by  $\sigma_i$  for up to three near-neighbour shells.

clear that the motions of adjacent sodiums and oxygens are strongly correlated in both glasses. Evidently the Na–O force constants are considerably stiffened by the SiO<sub>2</sub> environment and this explains the unusual strength of the Na–O band in the far IR absorption<sup>20</sup>. As in the case of the Si environment there is no real evidence for static disorder in the oxygen ligand around Na in the glasses.

The short-range order around sodium in crystalline silicates varies considerably with composition. Interestingly the local configurations revealed by EXAFS in the glasses are similar to those found in their crystalline polymorphs. In the binary sodium silicates Na<sub>2</sub>Si<sub>2</sub>O<sub>5</sub> (ref. 32) and Na<sub>2</sub>SiO<sub>3</sub> (ref. 33) all sodiums are fivefold coordinated in a distorted trigonal bipyramid of oxygen. The average bond length is 2.40 Å which is slightly longer than that of the glass (2.30 Å). In ternary silicates, on the other hand, the coordination is seldom as high as 5 and is usually 2 or 3 with a radius in the range 2.4–2.5 Å. The remaining oxygens are taken up in a broader outer shell (for example Na<sub>2</sub>CaSiO<sub>4</sub> (ref. 1) and Na<sub>2</sub>Mg<sub>2</sub>Si<sub>6</sub>O<sub>15</sub> (ref. 34) at separations that are non-bonding in the conventional picture of anion-cation contact.

Beyond the oxygen ligand in the range 3–4 Å in both binary and ternary crystalline silicates there are Si and modifier shells but these distributions are usually very broad. Given the large distribution of Si–O–Si bond angles in the glasses it is not surprising that a strong contribution from cation shells is absent in the  $\chi(E)$  for Na as it is for Si.

## Discussion

Since the ideas of Zachariasen<sup>3</sup> several approaches to the structure of glass have developed. These have been concerned with exploring the disordered topology which can incorporate the local order evident from the diffraction spectrum. For silicate glasses this is dominated by correlations in the silicon–oxygen network.

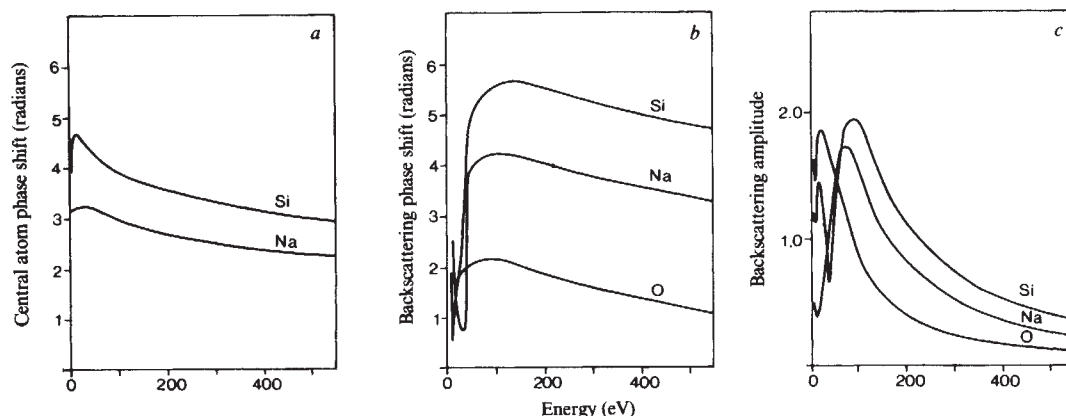
Specific CRN models have been built for silica<sup>4</sup> and also amorphous elemental semiconductors such as Ge (ref. 35), As (ref. 36) and Se (ref. 37). These have proved particularly successful both in predicting the experimental diffraction spectrum and in the structurally sensitive electronic and phonon properties<sup>38</sup>. Although these models mainly use single covalent interatomic interactions, in some instances attempts have been made

to introduce Van der Waals interactions<sup>36,37</sup>. In no case, however, in a covalent network have ionic interactions like those between modifying cations and oxygen been added. Indeed in the conventional Zachariasen picture the local structure of alkali and alkaline earth cations is left unspecified: they occupy a ‘statistical distribution’ of holes in the three-dimensional covalent network. Normal diffraction data have provided no strong evidence to the contrary.

Models constructed from a dense random packing of spheres (DRPs)<sup>39,40</sup> have provided an approach which initially was quite separate from the CRN idea. DRPs have been used to model amorphous elemental metals and alloys<sup>41–43</sup> and recently applied to silicate glasses. By treating the spheres as the oxygen lattice, the interstices and cavities have been explored as locations for silicons and modifying cations<sup>44</sup>. Indeed by using soft sphere packing, DRPs can be reduced to an assembly of distorted tetrahedra and octahedra<sup>45</sup>. But at best this is an idealized model although it does accord modifying cations a specific environment—octahedral.

Although minimum energy configurations have been orchestrated from the coordinates of hand-built models, both CRN and DRP models are essentially static. The alternative approach, therefore, has been to consider glass structure in relation to the structure of liquids. This has been concerned less with the topology of the connected lattice and more with relationships between the medium range order and the thermal and physical properties peculiar to the glassy state<sup>46–48</sup>. The outcome has been cluster-like structures. For silicate glasses these involve islands of SiO<sub>2</sub> with the modifying component taking up boundary locations. Within this area several interesting molecular dynamic calculations have been performed to model both silica<sup>49</sup> and sodium silicate glasses<sup>50</sup>. Although these assemblies are seldom large enough to say anything about the medium range order or indeed about the connectivity of the frozen glass, they do emphasize a relatively ordered oxygen ligand around sodium or calcium as well as the tetrahedral ligand around silicon.

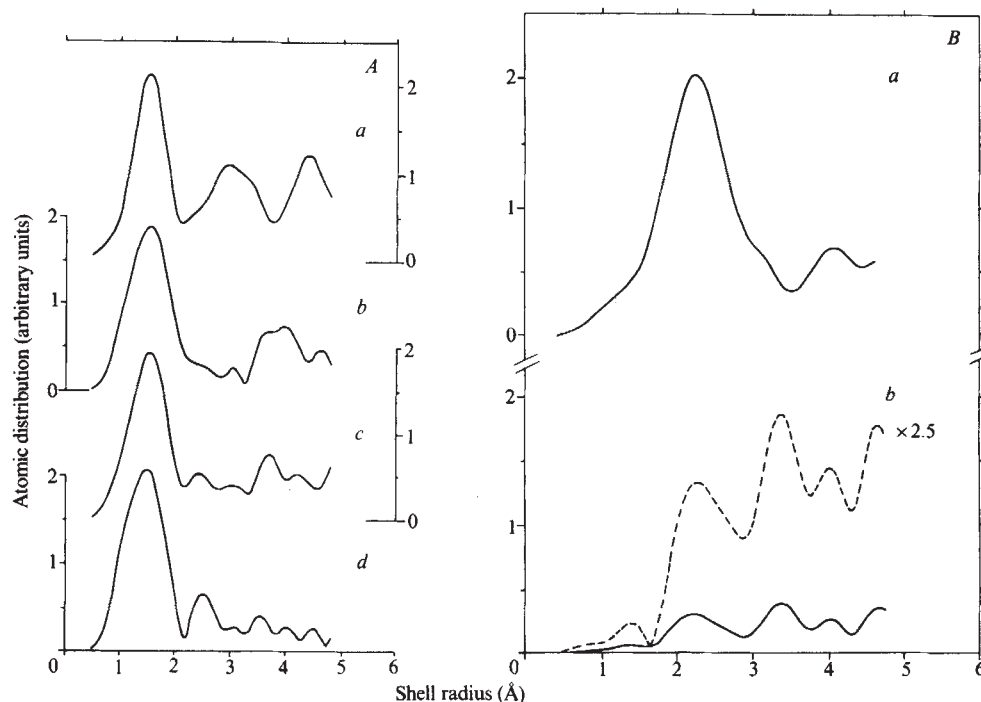
We have demonstrated that EXAFS furnishes an important missing ingredient required to specify the local structure of glass. We have presented here data for two silicate glasses, but we have also observed Na EXAFS in sodium diborate glass<sup>51</sup> and (at short wavelengths) Rb EXAFS in germinate glasses<sup>52</sup>. In all



**Fig. 3** Calculated atomic scattering factors used in the EXAFS analysis. *a*, Central atom ( $l=1$ ) phase shifts for Si and Na. *b*, Backscattering phase shifts for Si, Na and O. *c*, Backscattering amplitudes for Si, Na and O.



**Fig. 4** A, Atomic distributions  $F(r)$  about silicon for: a,  $\alpha$ -quartz; b, silica; c, sodium disilicate glass; d, soda-lime-silica glass. These were obtained by Fourier transforming the normalized EXAFS spectra given in Fig. 2. B, Atomic distributions  $F(r)$  about sodium for: a, sodium disilicate glass; b, soda-lime-silica glass. Following Gurman and Pendry<sup>18</sup> the back-scattering parameters for O were used and the central atom phase shifts for Si. Truncation effects were minimized by using a gaussian shaped window. This was centred at 122 eV—giving the best agreement for  $\alpha$ -quartz.



cases: the existence of extended fine structure confirms that the local structure of alkali cations is well-defined; and the fact that the spectrum varies from glass to glass indicates that the specific local order is a function of the composition. This should not be too surprising. In crystalline silicates for instance, whilst there is only one minimum energy configuration for silicon compared with many for sodium or calcium<sup>53</sup>, nevertheless, in a given silicate usually one particular configuration for each modifier persists—the local order is basically a function of stoichiometry and packing. Our present EXAFS results for glasses demonstrate the importance of the modifier environment in silicate chemistry.

We therefore envisage the structure of silicate glasses to be a natural extension of the structure of crystalline silicates. As such it will be made up of two interpenetrating sub-lattices: a covalent network consisting of the  $\text{SiO}_2$  component 'intercalated' by an ionic fraction made up of the modifier component (such as  $\text{Na}_2\text{O}$ ). In the crystalline state, silicates usually have a layer or chain structure with the modifying component binding these elements together<sup>53</sup>. In the glassy state both sublattices will be random. Whilst the vestiges of  $\text{SiO}_2$  layers and chains will be retained in the short range, in the long range we expect they will be lost into a three-dimensional CRN topology. Any correlations between these larger units will constitute the medium-range order predicted elsewhere<sup>48</sup>. The constraints on medium-range order will in effect be balanced by the degree of order in the random packed modifier component.

Furthermore we propose that for the present stable glass compositions both the covalent and the ionic sublattices will be continuous. Certainly the density of silicon or oxygen point

defects in annealed silicate glasses is no greater than in silica or quartz<sup>26</sup> which suggests the covalent sublattice is almost completely connected. In addition diffusion measurements of Na in silicate glasses<sup>54,55</sup> point to an interstitialcy transport mechanism. An ionic  $\text{Na}_2\text{O}$  sublattice could support this, in which case the observed correlated transport of Na could only be maintained if this lattice were sufficiently continuous to support percolation paths. This seems to be the case for a wide variety of glasses provided the concentration of  $\text{Na}_2\text{O}$  is  $>10$ – $20$  mole (ref. 55). (Crystalline  $\text{Na}_2\text{O}$  has an anti-structure fluorite<sup>1,51</sup> and as such has many cation interstitial sites.)

The structural link between the two sublattices in our model is effected through the so-called 'non-bridging' oxygens of Zachariasen<sup>3</sup>—something of a misnomer. In crystalline silicates these oxygens only singly coordinated to modifying cations usually form the ligand of the modifying cation: they are the anionic complement. We suggest this is also the function of 'non-bridging' oxygens in glasses. Local ordering of such oxygens around (in this case) sodium requires the ordering of sodiums around a non-bridging oxygen. The clustering of alkali and alkaline earth cations around non-bridging oxygens has often been suggested. It is a natural outcome of local order around modifiers and was predicted in earlier molecular dynamic calculations<sup>50</sup>.

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# Bacterial symbionts and low $^{13}\text{C}/^{12}\text{C}$ ratios in tissues of Pogonophora indicate unusual nutrition and metabolism

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*Observations on fine structure, metabolic enzymes and stable isotope ratios of several species of Pogonophora from a wide range of habitats suggest that members of this enigmatic phylum of worm-like deep-sea animals use internal chemoautotrophic bacteria as part of their nutrition, allowing them to exploit scarce nutrient and energy resources.*

ALL known representatives of the phylum Pogonophora lack mouth, digestive tract and anus<sup>1,2</sup>, and there has been much speculation about their food sources and feeding mechanisms<sup>3,4</sup>. Until recently, absorption of dissolved organic matter (DOM) through the epidermis was regarded as the simplest and most likely of the hypotheses offered, and experiments suggest that at least one species could exist solely by uptake of DOM from concentrations equivalent to those measured in sediment pore-water<sup>5</sup>. However, very recent studies on the related but much larger Vestimentifera show they possess enzymes indicative of autotrophy, probably located in endosymbiotic chemoautotrophic bacteria<sup>6,7</sup>. The giant vestimentiferans live close to hydrothermal vents in rift zones, where the water is rich in reduced inorganic compounds, especially sulphides<sup>8</sup>. This 'crustal' source is believed to be the energy supply that fuels the rich communities of animal life found at the vents<sup>9</sup>. Thus, symbiosis between the vestimentiferans and sulphide-oxidizing bacteria would be a further expression of the reliance of the community on a rich local input of energy.

In contrast to conditions at the vents, the small Pogonophora found in 'normal' habitats along the continental slope and in deep fjords live in oligotrophic conditions, and it has been difficult to understand how they get enough food at the low levels of organic input to sediments in deep water<sup>10</sup>. It is therefore of interest to report here preliminary results from a group of related investigations into fine structure, metabolic enzymes and stable isotope ratios of several species of small Pogonophora. The findings include the occurrence of bacteria in specialized tissues in the posterior half of the body; the presence of enzymes associated with fixation of  $\text{CO}_2$  by the Calvin cycle; and in the tissues and tubes, the lowest ratio of  $^{13}\text{C}$  to  $^{12}\text{C}$  yet reported for contemporary marine organic material. Taken together, these facts argue against reliance on particulate or dissolved organic matter as the only food source and indicate a more complex metabolism including chemoautotrophic symbiosis, possibly adapted for efficient recycling of scarce resources in oligotrophic habitats.

## Occurrence of bacteria

Electron microscopy of three species of *Siboglinum* (*S. atlanticum* from the Bay of Biscay, *S. ekmani* and *S. fiordicum* from the fjords near Bergen), whose capacity for epidermal uptake of DOM has been tested<sup>5</sup>, a new species of *Siboglinum* from the Skagerrak and the slightly larger *Oligobranchia gracilis* from the Bay of Biscay shows numerous bacteria in the post-annular region<sup>1,11</sup> of all five species. The bacteria are confined to tissue lying between the two longitudinal blood vessels, flanked by the lateral coelomic cavities. The anatomy of the tissue has been seen most clearly in *S. fiordicum* (Fig. 1): there is a hollow cylinder of bacterium-containing cells with a central lumen filled with coelomic fluid; a network of blood spaces permeates the cylinder wall; and an outer layer of peritoneal cells separates the bacterium-containing cells from the lateral coelomic spaces.

In *S. atlanticum*, *S. ekmani*, *S. fiordicum* and *O. gracilis*, the bacteria are rod-shaped, about 0.16–0.30  $\mu\text{m}$  in diameter and 1–3  $\mu\text{m}$  long (Fig. 2a, b). The cell wall consists of an outer layer, an intermediate layer and plasma membrane, typical of a Gram-negative bacterium<sup>12</sup>. The cytoplasm is moderately electron dense, but there is a lighter core containing some electron-dense strands, presumably DNA. In the cytoplasm there are small electron-dense particles and, occasionally, electron-lucent globules (Fig. 2a). The cells containing the bacteria are rich in glycogen and show unusual multilayered spherical granules 1–2  $\mu\text{m}$  in diameter (Fig. 2a). The bacteria are usually surrounded by host cell membrane, either individually or in groups inside a vacuole, and there are lysosome-like organelles containing degenerating bacteria (Fig. 2c). Immature and female pogonophores have much more of the bacteria-containing tissue than do males, in which most of the space in the post-annular region is filled with gonads and developing gametes.

In the new species of *Siboglinum* from the Skagerrak, the bacteria are larger and more rounded, up to  $3.2 \times 1.4 \mu\text{m}$ . They occur together with multilayered granules in cells which appear to proliferate from the muscle layer. In this species, the coelomic cavities are reduced to small spaces, some of them occupied by

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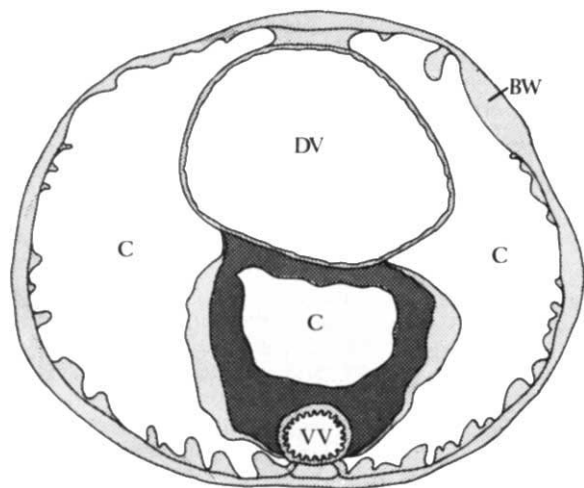


Fig. 1 Diagrammatic transverse section of the post-annular region of *S. fiordicum*. DV, dorsal vessel; VV, ventral vessel; C, coelom; BW, body wall. The bacterium-containing tissues are in heavy stipple.

tufts of cilia. Most of the bacteria are in membrane-bound vacuoles (Fig. 3), but some occur extracellularly between adjacent cells. The bacteria contain stacks of parallel membranes which are strikingly similar to structures reported from free-living bacteria which utilize methane<sup>13,14</sup>. Pinocytotic vesicles occur frequently inside and outside the bacterium-containing vacuoles, suggesting there may be metabolic exchanges between the bacteria and the host cells.

Recent studies on the giant vestimentiferan *Riftia pachyptila*<sup>15,16</sup>, which lives close to sulphide-rich hot vents in the floor of the east Pacific Ocean, show an abundance of globular bacteria, 3–5 µm in diameter, in trophosome tissue inside the main trunk of the animal<sup>7,17</sup>. Particles of elemental sulphur occur inside the cells containing these bacteria<sup>7,15</sup>; the bacteria are thought to be sulphide-oxidizing chemoautotrophs which may contribute substantially to the nutrition of their hosts<sup>6</sup>. *Riftia* and its close relative *Lamellibrachia* belong to the phylum Pogonophora but have many peculiarities and are considered a distinct sub-group<sup>15</sup>. However, the trophosome tissue in these species seems to be homologous with the bacterium-containing tissue of *Siboglinum* and *Oligobrachia*. The bacteria in *Riftia* are morphologically closer to the bacteria from the new species of *Siboglinum* found in the Skagerrak than to the rod-shaped organisms present in the other pogonophores studied.

Table 1 Fixation of <sup>14</sup>CO<sub>2</sub> by crude extracts of tissues of three species of Pogonophora at 8 °C

| Species                                               | Tissue wet (mg) | CO <sub>2</sub> fixation due to RuBP carboxylase (nmol per g wet wt per h) | CO <sub>2</sub> fixation due to other pathways in absence of RuBP |
|-------------------------------------------------------|-----------------|----------------------------------------------------------------------------|-------------------------------------------------------------------|
| <i>S. atlanticum</i> (whole animal)                   | 14.6            | 24.5                                                                       | 193.3                                                             |
| <i>S. fiordicum</i> (pooled anterior ends) (4)        | 4.24            | 20.1                                                                       | 6.8                                                               |
| <i>S. fiordicum</i> (pooled post-annular regions) (4) | 1.43            | 205.8                                                                      | 12.6                                                              |
| <i>S. ekmani</i> (pooled whole females) (5)           | 1.92            | 0                                                                          | 57.5                                                              |
| <i>S. ekmani</i> (pooled whole males) (5)             | 1.46            | 0                                                                          | 18.0                                                              |

To compare with previous experiments on *Riftia*<sup>18</sup>, frozen material, or fresh material quenched in liquid N<sub>2</sub>, was homogenized in 0.1 triethanolamine/HCl buffer, pH 7.3, and centrifuged at 24,000g for 10 min. The supernatant was tested for RuBP carboxylase (EC 4.1.1.39) by a modified <sup>14</sup>CO<sub>2</sub> technique<sup>53</sup>. Extracts of two additional specimens of *S. atlanticum*, which showed a positive qualitative reaction for RuBP carboxylase, were also tested for phosphoribulokinase (EC 2.7.1.19) by a spectrophotometric continuous assay<sup>54</sup>; they showed velocities of 0.23 and 0.39 µmol per g wet wt per min. The post-annular region of *S. fiordicum* contains most, but not all, of the bacteria.

## Fixation of carbon dioxide

In *Riftia pachyptila* the bacterium-containing trophosome tissues have been shown to possess two enzymes of the Calvin-Benson cycle of CO<sub>2</sub> fixation and three enzymes associated with sulphide oxidation, and it is suggested that the bacteria are chemoautotrophs which use the energy derived from oxidation of sulphide to fix CO<sub>2</sub> into organic compounds<sup>6,18</sup>. For comparison, three species of small pogonophores were tested for Calvin cycle enzymes (Table 1) and net uptake of CO<sub>2</sub> by all pathways was assessed to compare its nutritional potential with uptake of DOM (Table 2). Extracts of whole *S. atlanticum*, including part of the bacterium-containing tissues, reacted positively to tests for ribulose biphosphate (RuBP) carboxylase and phosphoribulokinase, but showed additional fixation by other pathways, presumably including carboxylation of phosphoenolpyruvate or pyruvate to oxaloacetate or malate<sup>19</sup>. With *S. fiordicum*, extracts of the anterior end showed low activity of RuBP carboxylase, equivalent to that in the extracts of *S. atlanticum*, whereas extracts of the post-annular region, where most of the bacteria occur, showed an order of magnitude more RuBP carboxylase activity and less fixation of <sup>14</sup>CO<sub>2</sub> by other pathways. RuBP carboxylase activity was not detected in extracts of the third species, *S. ekmani*, in which there was, nevertheless, a moderate fixation of <sup>14</sup>CO<sub>2</sub> by other pathways. The enzyme activities in the extracts of all the small pogonophores are lower than those found in extracts of the trophosome tissues of *Riftia*<sup>6</sup>, which can attain a rate of 13 µm per g wet weight per h. This difference may be due to dilution of the active cells by other tissues in the small pogonophores, from which it is not possible to dissect predominantly bacterium-containing tissues equivalent to the trophosome of *Riftia*. In the latter species such tissue forms a larger proportion of the body.

Total fixation of CO<sub>2</sub> in live *S. fiordicum* varied little between the anterior end and the post-annular region (Table 2), despite their relative differences in RuBP carboxylase activity *in vitro*. The maximum CO<sub>2</sub> fixed per gramme live weight was of the same order of magnitude as the fixation by homogenized extracts expressed on a wet weight basis, but comparisons of this

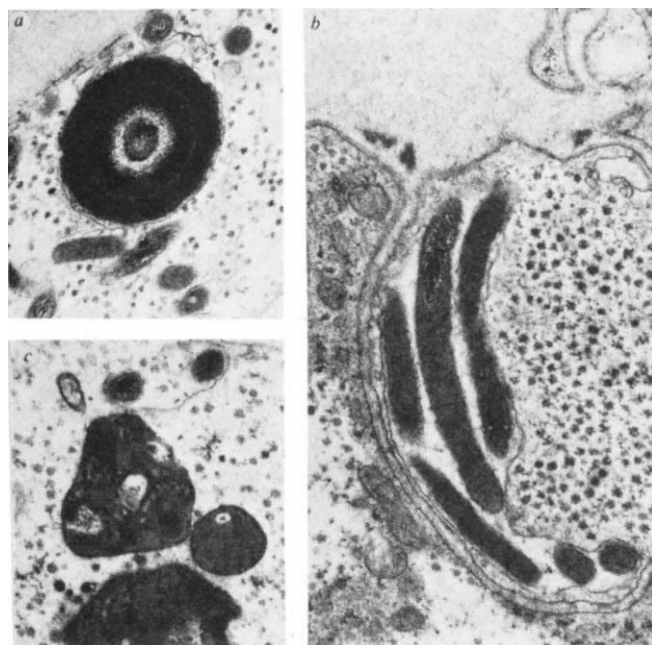


Fig. 2 Transmission electron micrographs of bacteria in cells of *S. fiordicum*. a, Bacteria (transverse and oblique section) and a multilayered granule; note blood space at upper left. ×20,500. b, Bacteria (LS and TS); note blood space above and between cells. ×22,600. c, Lysosomes containing degenerating bacteria. ×22,600. Methods: a, fixed 2.5% glutaraldehyde in 0.1 M phosphate buffer plus 0.6 M sucrose; b, c, fixed 2.5% glutaraldehyde in seawater. All were postfixed in OsO<sub>4</sub>, embedded in Spurr resin and the sections stained with uranyl acetate and lead citrate.

**Table 2** Fixation of  $^{14}\text{CO}_2$  by single live *Siboglinum fiordicum* at 6 °C

| Time (min)          | Mean wet<br>wt (mg) | Oxygenated seawater                              |     |     |                                                        | Deoxygenated<br>seawater,<br>also<br>containing<br>132 µg/l<br>of<br>sulphide |     |
|---------------------|---------------------|--------------------------------------------------|-----|-----|--------------------------------------------------------|-------------------------------------------------------------------------------|-----|
|                     |                     | Containing<br><sup>14</sup> CO <sub>2</sub> only |     |     | Also<br>containing<br>5.5 µM<br><sup>3</sup> H-glucose |                                                                               |     |
|                     |                     | 10                                               | 20  | 60  | 10                                                     | 60                                                                            | 60  |
| Anterior end        | 1.08                | 83                                               | 142 | 282 | 47                                                     | 227                                                                           | 125 |
| Post-annular region | 0.68                | 90                                               | 123 | 290 | 47                                                     | 105                                                                           | 186 |

Values are expressed as nM accumulated per g wet wt tissue. The animals were dissected out of their tubes and exposed to filtered seawater containing  $\text{NaH}^{14}\text{CO}_3$ . After the experiment they were divided into anterior and post-annular sections and the  $^{14}\text{C}$  fixed into organic compounds was evaluated as a fraction soluble in 80% ethanol and the remainder bound to the tissues<sup>23</sup>. Each fraction was freed of inorganic carbonate by acidification with 1 M HCl followed by heating to dryness at 50 °C under reduced pressure. The two fractions are pooled here. Concurrent experiments were made to check uptake of  $^{14}\text{C}$ - and  $^3\text{H}$ -labelled DOM for comparison with previous results<sup>23</sup>. The post-annular section contains most, but not all, of the bacteria.

**Table 3** Comparison of rate of fixation of  $^{14}\text{CO}_2$  with rate of uptake of  $^{14}\text{C}$ -labelled dissolved compounds in *S. fiordicum*

|                                | Environmental concentration (µmol l <sup>-1</sup> ) | Rate of uptake (nmol g <sup>-1</sup> h <sup>-1</sup> ) | Carbon equivalents (ng at g h <sup>-1</sup> ) |
|--------------------------------|-----------------------------------------------------|--------------------------------------------------------|-----------------------------------------------|
| Mean fixation of $\text{CO}_2$ | 208*                                                | 286                                                    | 286                                           |
| All amino acids <sup>23</sup>  | 14                                                  | 186                                                    | 558†                                          |
| Fatty acids <sup>23</sup>      | 6                                                   | 18                                                     | 54†                                           |
| Glucose <sup>23</sup>          | 6                                                   | 2                                                      | 12                                            |

\* Assumed to be the same as high-salinity oceanic water.

† A conservative estimate based on an average of 3 carbon atoms per molecule.

nature are difficult because activity of RuBP carboxylase may be lowered *in vitro*<sup>20</sup>. Fixation of  $\text{CO}_2$  was apparently depressed by the presence of glucose at environmental levels (6 µM), the greatest effect being seen in the post-annular region, and also by conditions of low oxygen and sulphide, when the greatest depression was at the anterior end. The post-annular region showed similar fixation into the soluble component whether oxygen was present or not, suggesting that it is the subsequent metabolism that is affected by the simulated anoxic conditions rather than the initial fixation. Uptake of glucose took place at rates similar to those established previously, and was not significantly reduced by 1-h exposures to simulated anoxic conditions.

Tables 1 and 2 show that the small pogonophores, like their giant relatives from the hydrothermal vents, do possess enzymes involved in fixation of  $\text{CO}_2$  via the Calvin cycle, and that this activity is greatest in the region of the body containing most bacteria. However, as already noted, they are also capable of incorporation of  $\text{CO}_2$  by other pathways. Marine invertebrates from at least 14 phyla can fix  $\text{CO}_2$  into the acids of the Krebs cycle<sup>21</sup>, and it is not surprising that Pogonophora have this ability; the rates calculated fall into the lower end of the range of  $\text{CO}_2$  uptake by other animals<sup>22</sup>. Table 3 compares the rate of fixation of  $\text{CO}_2$  by all pathways with the previously determined rate of DOM uptake<sup>23</sup>. *S. fiordicum* seems to obtain greater potential gain of carbon by uptake of DOM than by fixation of  $\text{CO}_2$ . The dissolved organic compounds can supply 73% of the minimum metabolic needs and addition of organic matter derived from fixation of  $\text{CO}_2$  would bring the total contribution to >100%.

## Stable carbon isotope ratios

The natural  $^{13}\text{C}/^{12}\text{C}$  ratio in the tissues of an animal is approximately equal to the carbon isotope composition of its food<sup>24–27</sup>; this feature has been used to investigate the nutrition of aquatic invertebrates<sup>27–34</sup>. For example, the  $^{13}\text{C}/^{12}\text{C}$  of the vestimentiferan *R. pachyptila* ( $\delta^{13}\text{C}$ –11‰) is unlike that of animals whose food is derived ultimately from open ocean photosyn-

thesis<sup>34</sup>, excluding straightforward heterotrophy as the primary means of nutrition. Together with other evidence, the isotope values point to other nutrient sources, including internal chemoautotrophy<sup>18</sup>. It was believed that measurement of the stable carbon isotope ratios in related pogonophores from less exotic environments might elucidate further the nutrition of these unusual animals; Table 4 gives the results and methods.

Carbon from the organic fraction of the sediment samples has a  $\delta^{13}\text{C}$  of about –20.5‰, a value typical of most marine sediments<sup>35</sup>. The  $\delta^{13}\text{C}$  values of the polychaetes (–16.1 to –18.5‰) are somewhat higher, a feature observed in other benthic communities and attributable to slight enrichment of  $^{13}\text{C}$  in the tissues as a function of trophic level<sup>29</sup>. In the case of *Eunice floridana*, which had the highest  $\delta^{13}\text{C}$  value, it is likely that its food source is the coral *Lophelia prolifera*, with which this worm is always associated. Thus *E. floridana*'s use of sedimentary organic carbon would be less direct than that of the deposit-feeding terebellid worms, perhaps explaining why the  $\delta^{13}\text{C}$  of the latter more closely approximates to that of the sediment (Table 4). Whatever the explanation for the polychaete worms, the carbon isotope ratios in their tissues and in the sediments bear little resemblance to those of the co-inhabitant *S. atlanticum*. The  $\delta^{13}\text{C}$  of this animal's tube and soft tissues ranged from –42.9 to –45.9‰, the lowest  $\delta^{13}\text{C}$  values yet reported for contemporary marine organic material<sup>35</sup>. The tube samples gave the most elevated values in this range, perhaps due to isotope fractionation effects associated with biosynthesis of the compounds composing the tubes.

If the demonstrated similarity between the  $\delta^{13}\text{C}$  of an invertebrate's food and its biomass<sup>24–27</sup> is also valid for

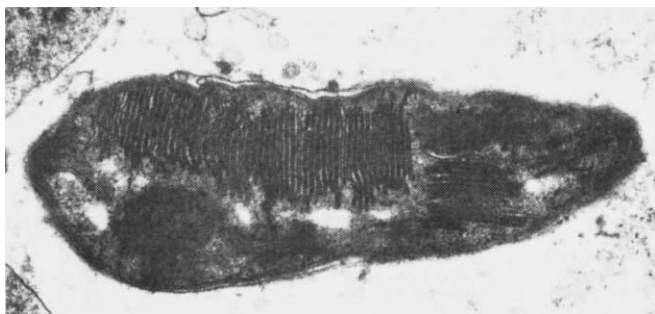
**Table 4** Values of  $\delta^{13}\text{C}$  for animals and sediment samples collected from the northern Bay of Biscay in July 1980

| Sample                                                                                      | Depth (m)   | Position                                   | $\delta^{13}\text{C}$ ‰ |
|---------------------------------------------------------------------------------------------|-------------|--------------------------------------------|-------------------------|
| 3 <i>S. atlanticum</i> in tubes                                                             | 1,850       | St. no. 1/11<br>47° 49.3' N<br>8° 8.9' W   | { –45.9<br>–45.8        |
| 3 Empty tubes <i>S. atlanticum</i>                                                          | 1,850       | St. no. 1/11<br>47° 49.3' N<br>8° 8.9' W   | { –43.3<br>–42.1        |
| 3 <i>S. atlanticum</i> , out of tubes                                                       | 1,530–1,810 | St. no. 1/15<br>47° 50.5' N<br>8° 5.8' W   | –45.3                   |
| 3 Terebellid polychaetes (2 <i>Pista</i> sp., 1 <i>Terebellides</i> )                       | 1,840–1,950 | St. no. 1/14<br>47° 50.0' N<br>8° 7.7' W   | { –18.3<br>–18.5        |
| 1 <i>Eunice floridana</i> , anterior musculature and body wall                              | 1,530–1,810 | St. no. 1/15<br>47° 50.7' N<br>8° 5.8' W   | { –16.1<br>–16.1        |
| Mud from <i>S. atlanticum</i> habitat                                                       | 1,840–1,950 | St. no. 1/14<br>47° 50.0' N<br>8° 7.7' W   | –20.4                   |
| Mud from <i>S. atlanticum</i> habitat                                                       | 1,650–1,850 | St. no. 1/20<br>47° 49.4' N<br>8° 8.9' W   | –20.6                   |
| 10 <i>S. atlanticum</i> (kept alive) in oxygenated water 9 days before preparation          | 1,730–2,180 | St. No. 2/20<br>48° 28.6' N<br>10° 20.1' W | { –44.6<br>–44.8        |
| Other marine organic material for comparison:                                               |             |                                            |                         |
| Benthic and pelagic animal tissues (refs 27–34 and refs therein)                            |             |                                            | –8 to –33.6             |
| Sedimentary organic material, various particle sizes and biochemical fractions (refs 35–44) |             |                                            | –8 to –38               |

$$\delta^{13}\text{C} = \left[ \left( \frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}}}{^{13}\text{C}/^{12}\text{C}_{\text{PDB standard}}} \right) - 1 \right] \times 10^3 (\text{‰})$$

Depths are uncorrected PDR soundings; where multiple echoes were recorded from the canyon walls the minimum and maximum are given. Specimens were collected in July 1980 by dredge, and dried at 50 °C under reduced pressure. Some were dissected out of the tube, others were dried *in situ* in the tube, which consists of a chitin–protein complex<sup>11</sup>. Dissected tissues of polychaete worms and samples of mud were dried in the same way. At UCLA the samples or subsamples were heated to dryness in 10 M HCl to remove inorganic carbon. The organic carbon remaining in 5–50-mg subsamples of the acid-treated material was completely oxidized to  $\text{CO}_2$  gas by the method of Stump and Frazer<sup>35</sup>. The relative  $^{13}\text{C}/^{12}\text{C}$  of these gas samples, representing the original organic carbon, was determined with a Varian MAT 250 ratio mass spectrometer.





**Fig. 3** Transmission electron micrographs of bacteria in cell of *Siboglinum* (new species from the Skagerrak).  $\times 28,500$ . Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer plus 0.6 M sucrose, postfixed in  $\text{OsO}_4$ , embedded in Araldite and sections stained with uranyl acetate and lead citrate.

pogonophores, the  $^{13}\text{C}$ -depleted condition of *S. atlanticum* indicates that its food source is quite unlike that of the associated polychaetes and markedly different from other marine animals previously analysed. Preliminary carbon isotope ratios of two further species of pogonophores from the Norwegian fjords also show considerable depletion of  $^{13}\text{C}$ . A  $\delta^{13}\text{C}$  of  $-45.3\%$  for *S. ekmani* from 700 m agrees well with the values for *S. atlanticum*, and suggests a similar carbon/food source. A somewhat lesser depletion of  $-35.3\%$  found in *S. fiordicum* living in mud of greater organic content at 30 m is still difficult to reconcile with a sedimentary carbon source. In all these examples, given the typical  $\delta^{13}\text{C}$  ( $\sim -20\%$ ) of the organic material in the sediment, it is difficult to identify, from the spectrum of organic materials likely to be present, a candidate that could be consumed directly to produce a tissue  $\delta^{13}\text{C}$  lower than  $-40\%$ . Numerous isotopic analyses of dissolved and particulate sedimentary materials, including amino acids, carbohydrates and lipids, show a wide range of carbon isotope abundances<sup>35-44</sup> but except possibly the low molecular weight hydrocarbons, there is nothing equivalent to the  $\delta^{13}\text{C}$  of the tissues of *Siboglinum atlanticum* and *S. ekmani*.

## Discussion

The carbon isotope analyses indicate that the small pogonophores are unlikely to rely entirely on direct utilization of particulate or dissolved carbon derived from surface photosynthesis. The presence of bacteria, and of Calvin cycle enzymes which have not been found in animals and are presumed to be located in the bacteria, point to chemoautotrophy as a possible source of nutrition. There are several possible explanations of the low  $\delta^{13}\text{C}$  reported here for Pogonophora from 'normal' habitats. The first supposes that the major carbon source is  $\text{CO}_2$ , which is fixed by the bacteria operating in a chemoautotrophic mode. There is a large discrimination against  $^{13}\text{CO}_2$  and in favour of  $^{12}\text{CO}_2$  during RuBP carboxylation, which in equilibrium conditions with atmospheric  $\text{CO}_2$  produces autotroph biomass whose  $\delta^{13}\text{C}$  is rarely lower than  $-30\%$  (ref. 35). However, if  $^{13}\text{C}$ -depleted respired  $\text{CO}_2$  ( $\sim -20\%$ ) was used rather than, or in addition to, dissolved surface sea sources (0 to  $-7\%$ ), then resulting tissue  $\delta^{13}\text{C}$  values could be considerably lower than  $-30\%$ . Such biological refixation and recycling of carbon and subsequent isotope fractionation have been suggested to explain the  $\delta^{13}\text{C}$  values of  $-40$  to  $-47\%$  found in insects and plankton from a sub-alpine lake<sup>30,45</sup>, as well as even lower

values found in one example in Upper Pleistocene organic materials<sup>46</sup>. A similar process, whereby  $^{13}\text{C}$ -depleted  $\text{CO}_2$  from benthic respiration or from internal pogonophoran respiration is fixed by the endosymbionts, could explain why the  $^{13}\text{C}$  depletion in the pogonophores analysed is so unlike all but those of the organisms noted above. In addition, the incorporation of  $\text{CO}_2$  into cell carbon by pathways other than RuBP carboxylation, as suggested by our studies, could contribute to the unusual isotope abundances observed. The few chemosynthetic bacteria studied appear to prefer  $^{12}\text{CO}_2$  to a greater extent than do photoautotrophs<sup>47,48</sup>, but little work has been done on the cause of this isotope effect or on its prevalence in nature.

With regard to internal recycling of  $\text{CO}_2$ , the extent of isotope fractionation would depend on the nature of the supposed symbiotic relationship between the pogonophore and the internal bacteria. A recent theoretical study of nutrient relationships between shallow-water invertebrates and symbiotic algae predicts that where growth-limiting nutrients are scarce and concentrated in organic matter, production by a symbiotic system could be 2 or 3 orders of magnitude greater than that of a system with separate autotrophs and heterotrophs<sup>49</sup>. If similar arguments could be applied to the pogonophore-bacteria association, and if there was efficient recycling, continued re-use by the bacteria of carbon dioxide produced by the pogonophore might explain the isotope depletions observed.

The unusual carbon isotope ratios of *Siboglinum* might also result from utilization of methane as a carbon source. Methane generated from anaerobic sediments typically has  $\delta^{13}\text{C}$  values lower than  $-40\%$  (ref. 35). Presumably microorganisms capable of incorporating methane into cell material would be comparably depleted in  $^{13}\text{C}$ . Such microbial methanotrophy could provide a source of food for higher organisms whose tissue carbon, as a consequence, would possess  $\delta^{13}\text{C}$  values similar to those observed in *Siboglinum*. If endosymbiotic bacteria were incorporating carbon from methane, this process would not necessarily preclude  $\text{CO}_2$  fixation or assimilation of DOM, because all these processes can be quantitatively significant in obligate methanotrophs<sup>14</sup>. Furthermore, the presence of RuBP carboxylase in extracts of *Siboglinum* is not inconsistent with this hypothesis as some methanotrophic bacteria are now known to be capable of autotrophy via the Calvin cycle<sup>50,51</sup>.

The above hypotheses are not mutually exclusive; there could be a degree of mixotrophy as already suggested<sup>52</sup>, the relative importance of each process being governed by environmental levels of DOM, reduced inorganic compounds and dissolved low molecular weight hydrocarbons. It remains to be discovered how the bacteria in the small pogonophores obtain their energy, and, in particular, whether they can make use of the relatively low sulphide and methane content of the reducing deposits in which the pogonophores live.

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# Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene

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*By replacing either the early or the late genes of SV40 with a cloned copy of the influenza virus haemagglutinin gene we have constructed recombinant viruses which, in infected cells, express large quantities of haemagglutinin. This glycoprotein, over 10<sup>8</sup> molecules of which are produced per cell, is identical in molecular weight to authentic influenza virus haemagglutinin, accumulates at the cell surface and displays haemabsorbing activity.*

THE amino acid sequences of a number of integral membrane proteins have recently been determined, either directly by protein chemistry<sup>1–6</sup> or by analysis of the DNA sequences of cloned versions of their genes<sup>7–15</sup>. The proteins include those found naturally on the surface of cells as well as virus-coded proteins whose biosynthesis utilizes cellular enzymes and processes for their translation, membrane translocation and transport, glycosylation and maturation. These viral membrane proteins thus provide useful model systems for the study of the structure, function and biosynthesis of integral membrane proteins in general.

Of all such membrane proteins, the haemagglutinin (HA) glycoprotein of influenza virus is the best characterized. The complete amino acid sequences of HAs from many influenza strains are known<sup>5–13</sup>, the three-dimensional structure of the glycoprotein spike has been determined by X-ray crystallography<sup>16</sup> and the major antigenic regions of the molecule have been defined<sup>17</sup>.

A characteristic of many integral membrane proteins is that they contain hydrophobic amino acid sequences at their amino and carboxyl termini. It is widely believed that the amino-terminal 'signal' peptide functions in attachment to and translocation of the nascent polypeptide chain across the endoplasmic reticulum<sup>18</sup> while the carboxyl-terminal sequence serves to anchor the completed proteins in the cell membrane<sup>19,20</sup>. Perhaps surprisingly, comparison of the structures of membrane proteins in general and of the set of HA molecules in particular reveals that the hydrophobic sequences at the amino and carboxyl termini are not highly conserved<sup>8</sup>. One interpretation is that hydrophobicity rather than specific amino acid sequence is important in the interaction of amino and carboxyl termini with membrane components. However there are not yet enough data to deduce how such interactions might occur.

One approach to the study of integral membrane proteins is to introduce alterations in their amino acid sequences and to analyse the subsequent effects on the biosynthesis, structure and

function of the molecules. It would be of particular interest to determine the effects of alterations in the hydrophobic sequences on the membrane interaction and translocation. The wealth of information on HA makes it an ideal candidate for such studies. However, because this protein is naturally encoded by an RNA genome, it has not been feasible to manipulate its primary structure by directed alteration of the nucleotide sequence. To circumvent this difficulty, we decided to introduce mutations into the cloned DNA copy<sup>8</sup> of the influenza virion RNA segment which encodes HA. The first stage in this process is to synthesize HA from the cloned gene, and we describe here the construction and use of recombinant viral vectors which express large amounts of HA at the cell surface of eukaryotic cells.

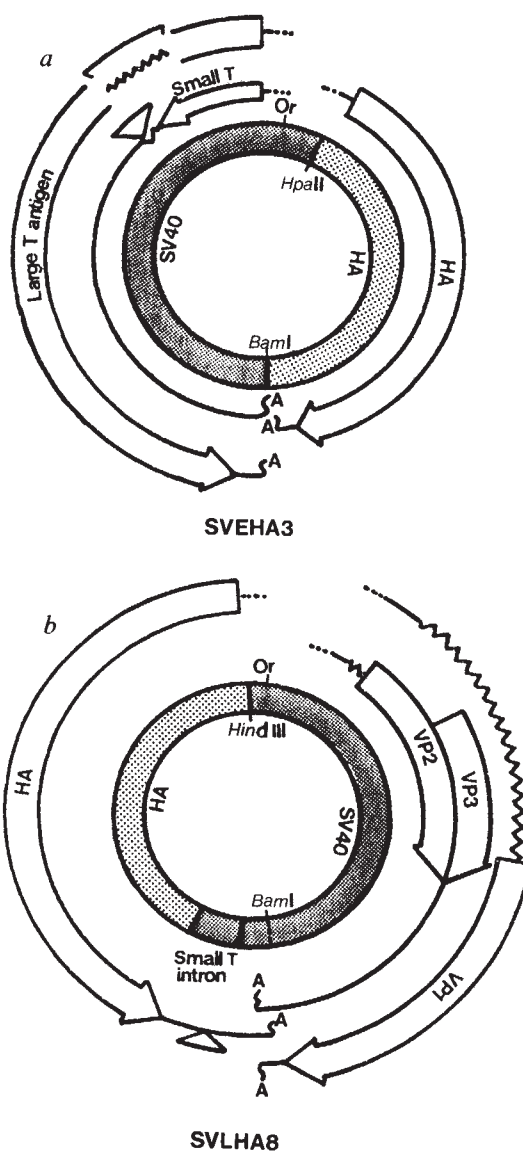
## Construction of vectors

The HA gene from influenza virus strain A/Japan/305/57 (H2N2) has previously been cloned in the plasmid vector pAT153 and its complete nucleotide sequence has been determined<sup>8</sup>. It contains no intervening sequences and is a convenient size for insertion into the late or the early region of the SV40 genome. Because the HA gene contains no promoter of its own, the vectors were designed so that HA was expressed under the control of the SV40 early or late promoters. The details of the constructions are given in Fig. 1 legend.

To construct late-replacement vector SVEHA3 the HA gene was inserted into the late region of SV40 between the *Hpa*II site at nucleotide 346 and the *Bam*HI site at nucleotide 2,533 (SV numbering system)<sup>21</sup>. This recombinant viral genome was cloned into the *Bam*HI site of pAT153 and propagated in *Escherichia coli*. For transfection into simian cells, the recombinant SV40–HA genome was excised from the plasmid by digestion with *Bam*HI, purified and ligated to yield the structure SVEHA3 shown in Fig. 1. This recombinant viral genome contains the SV40 origin of DNA replication and an intact set of early genes. The HA gene is joined to the noncoding sequences which would normally be transcribed into the untranslated 5'



**Fig. 1** SV40-HA recombinant vectors designed to express haemagglutinin in simian cells. The shaded circles represent the double-stranded circular DNA genomes of the recombinant viruses. The protein-coding sequences are indicated by blocked arrows. Untranslated parts of the mRNA are shown as solid lines; dots indicate uncertainty about the exact position of the 5' end of the mRNA; zigzag lines show segments spliced out; a wavy line with an A illustrates the 3'-terminal poly(A) tract. (After ref. 42.) **a**, SV40 late region replacement vector (SVEHA3). Plasmid pJHA<sup>9</sup> was linearized with *Hind*III. (All restriction endonuclease digestions were carried out using the conditions recommended by New England Biolabs.) The homopolymeric tails at the 5' end of the HA gene were removed using the double-stranded exonuclease, *Bal*31<sup>54</sup> (Bethesda Research Labs). Five  $\mu$ g of linearized pJHA, dissolved in 50  $\mu$ l of 12 mM CaCl<sub>2</sub>, 12 mM MgCl<sub>2</sub>, 0.6 M NaCl, 20 mM Tris-Cl pH 8.1, 1 mM EDTA, 200  $\mu$ g ml<sup>-1</sup> bovine serum albumin (BSA), were incubated at 30°C with 0.25 U of *Bal*31. Aliquots were removed after 1, 1.5 and 2 min into an equal volume of ice-cold 0.1 M EDTA. The samples were then pooled and the DNA purified by extraction with phenol and precipitation with ethanol. The DNA was dissolved in 20  $\mu$ l of 60 mM Tris-HCl, pH 7.5, containing 8 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol, 1 mM ATP, 0.2 mM dNTPs and 1 U of the Klenow fragment of *E. coli* DNA polymerase was added. After incubation for 30 min at 20°C, the DNA was again purified by phenol extraction and ethanol precipitation. *Hind*III linkers (Collaborative Research) were added to the resulting population of molecules as described previously<sup>43</sup>. After cleavage with *Hind*III and *Bam*HI, the fragments of HA DNA (~1,800 nucleotides long) were purified by polyacrylamide gel electrophoresis and cloned between the *Hind*III and *Bam*HI sites of pAT153. Clones lacking the homopolymer tails were identified by sizing of restriction endonuclease fragments of DNA. The nucleotide sequences adjacent to the *Hind*III linkers in several clones were determined using the Maxam-Gilbert technique<sup>44</sup>. Clone pJHB16 was selected in which the nucleotide immediately following the *Hind*III linker was the A of the initiation codon of the HA coding sequence. pJHB16 was linearized by digestion with *Hind*III and the protruding tails were filled in using the Klenow fragment of DNA polymerase as described above. The DNA was then digested with *Bam*HI and the fragment containing the HA gene was purified by gel electrophoresis. Similarly the plasmid pJYM6<sup>45</sup>, which contains the entire SV40 genome cloned through its *Bam*HI site, was digested with *Hpa*II and the protruding tails were repaired as before. After digestion with *Bam*HI, the 3,039 nucleotide fragment was purified and ligated to the 1,800 nucleotide HA fragment and to plasmid pAT153 which had been linearized with *Bam*HI and treated with calf intestinal phosphatase<sup>46</sup> to remove 5'-terminal phosphates. After transfection into *E. coli* HB101, recombinant plasmids containing HA and SV40 sequences were identified by hybridization using the Grunstein-Hogness technique<sup>47</sup> and their structures were verified by restriction endonuclease digestion. The DNA of one of these recombinant plasmids was then digested with *Bam*HI and the SV40-HA fragment was purified by gel electrophoresis. Circularization of this fragment by ligation at low DNA concentration (3  $\mu$ g ml<sup>-1</sup>) produced the recombinant vector SVEHA3 shown above. This was introduced into CV-1 cells together with the SV40 early deletion mutant *dl*1055<sup>23</sup> using DEAE dextran<sup>24</sup> (75 ng each DNA per 10<sup>6</sup> cells). After 5 days at 37°C, the cells and medium were frozen and thawed three times and fresh monolayers of CV-1 cells were infected with 0.3 ml of the resulting extract. This was repeated twice to obtain a virus stock. **b**, SV40 early region replacement vector (SVLHA8). A segment of SV40 DNA which contains the intron of the small T antigen gene linked directly to the poly(A) addition site of the same gene (unpublished data) was inserted into pJHB16 at the *Bam*HI site located in noncoding sequences after the 3' end of the HA gene. The resulting 2,600 nucleotide fragment consisting of the HA gene and the composite SV40 segment was obtained by digestion with *Hind*III and *Bam*HI and purified by gel electrophoresis. This DNA was ligated to a fragment obtained by partial digestion of *Bam*HI-linearized SV40 DNA with *Hind*III at nucleotide 5,171<sup>21</sup>. The partial digestion product contains the early promoter, the origin of DNA replication and the viral late genes. The resulting recombinants were cloned into plasmid pAT153 which, as before, had been linearized with *Bam*HI and treated with calf intestinal phosphatase<sup>46</sup>. After transfection into *E. coli* HB101, the recombinant plasmids containing HA and SV40 sequences were identified by hybridization using the Grunstein-Hogness technique<sup>47</sup> and their structures were verified by restriction endonuclease digestion. The DNA of one of these recombinant plasmids was then digested with *Bam*HI and the SV40 HA fragment was purified by gel electrophoresis. Circularization of this fragment by ligation at low DNA concentration (3  $\mu$ g ml<sup>-1</sup>) produced the recombinant vector SVLHA8 shown above. This recombinant was introduced into COS-1 cells<sup>29</sup> using DEAE-dextran<sup>24</sup> and was propagated in these cells in the absence of helper virus.



region of SV40 late mRNAs. Late in infection of permissive simian cells this recombinant should express a hybrid mRNA consisting of untranslated SV40 sequences at its 5' end and an intact set of HA coding sequences. Polyadenylation could occur either at a site which has been postulated to function during the synthesis of HA mRNA from its natural influenza viral RNA template or at the normal site for late SV40 mRNAs at nucleotide 2,674<sup>21</sup>. Translation of this hybrid mRNA should begin at the first AUG which marks the beginning of the HA coding sequences.

**Early replacement vector SVLHA8:** A segment of DNA was constructed which contained the intron of the small T antigen gene of SV40 linked directly to the poly(A) addition site derived from the same gene. This segment was linked to the HA gene and the composite fragment inserted into the SV40 genome between the *Hind*III site at nucleotide 5,171 and the *Bam*HI site at nucleotide 2,533 (ref. 21). The recombinant viral genome was cloned into the *Bam*HI site of pAT153 and propagated in bacteria. For transfection into simian cells, the recombinant

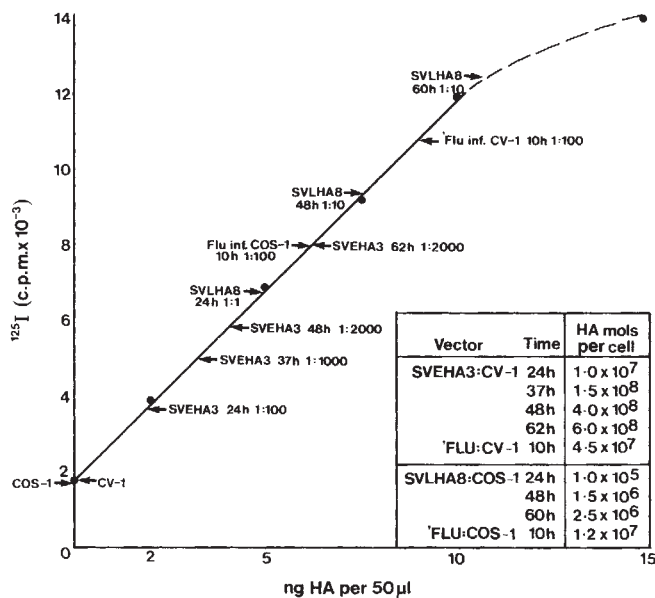
SV40-HA genome was excised from the plasmid by digestion with *Bam*HI, purified and ligated to yield the structure SVLHA8 shown in Fig. 1. This recombinant viral genome contains the SV40 origin of DNA replication and an intact set of late genes. The HA gene lies downstream from the early promoter within the untranslated sequences which normally precede the sequences coding for SV40 early gene products. Transcription from this promoter should therefore generate hybrid mRNAs which begin at the usual major sites in SV40 DNA<sup>22</sup> (nucleotides 5,320 and 5,326) and consist of ~70 nucleotides of untranslated SV40 sequence linked to the entire coding sequence of HA. Polyadenylation could occur either at the postulated site within the 3'-untranslated sequences of the HA gene or at the normal site for early SV40 mRNAs at nucleotide 2,587 (ref. 21). In the latter case there is an opportunity for a spliced mRNA to be formed by removal of the SV40 small T intron which is inserted into the construct downstream of the HA sequences and upstream from the SV40 early poly(A) addition site.

## Expression of HA by the late replacement vector SVEHA3 in CV-1 cells

Because the vector SVEHA3 contains an intact copy of the gene coding for SV40 large T antigen, its DNA will replicate efficiently in permissive simian cells. However, production of infectious virions containing the recombinant genome requires complementation by a helper virus such as *dl1055* (ref. 23), an early deletion mutant of SV40 which will supply SV40 capsid proteins for the assembly of virions.

SVEHA3 and *dl1055* DNAs were introduced together into CV-1 cells using DEAE-dextran<sup>24</sup>. The virus produced by these cells was serially passaged twice on fresh monolayers. At this stage a virus stock was obtained which induced a total cytopathic effect within 72 h after infection of CV-1 cells.

Monolayers of CV-1 cells were infected with this virus stock, and at various times later cell extracts were prepared and assayed for HA antigen by a solid-phase radioimmunoassay<sup>25</sup>. Figure 2 shows that the amount of HA antigen progressively increased with time so that by 62 h after infection, an average of  $6 \times 10^8$  molecules per cell was present. This is equivalent to 80  $\mu$ g of HA per 90 mm dish of cells.



**Fig. 2** Analysis of HA in cell extracts by solid-phase radioimmunoassay. Just-confluent monolayers of CV-1 cells or COS-1 cells were mock-infected or infected with SVEHA3 or SVLHA8 respectively as described previously<sup>48</sup> using a 1:8 dilution of the virus stocks. Infection of cells with the A/Japan/305/57 strain of influenza virus ( $10^3$  plaque-forming units per cell) was carried out as described by Hay<sup>49</sup>. At various times after infection the medium was aspirated, and the cell monolayers were washed once with cold Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.4). The cells were then scraped from the dish using a policeman, pelleted by centrifugation at 2,000 r.p.m. for 5 min and lysed in 300  $\mu$ l per 90 mm dish of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 1% NP40). After brief vortexing and standing for 20 min on ice, the nuclei and cell debris were removed by centrifugation for 1 min at 10,000 r.p.m. Radioimmunoassays were performed by a modification of a procedure described previously<sup>25</sup>. Rabbit anti-HA IgG was purified by chromatography on Protein A-Sepharose<sup>53</sup>. Aliquots (50  $\mu$ l) of the anti-HA IgG (50  $\mu$ g ml<sup>-1</sup> in 0.2 M NaHCO<sub>3</sub>) were dispensed into the wells of PVC microtitre plates and the antibody was allowed to adsorb for 20 min at room temperature. The antibody solution was then aspirated and the wells washed three times with wash buffer (phosphate-buffered saline (PBS) A containing 0.5% calf serum, 0.1% BSA and 0.1% NP40). Cell extracts were diluted as necessary in lysis buffer. Triplicate samples of the extracts or of known amounts (1–15 ng) of purified viral HA<sup>50</sup> (supplied by Dr John Skehel) were added to the wells and the plates were incubated at room temperature for 1–2 h. The samples were aspirated and the wells washed as before. Aliquots (50  $\mu$ l, 200,000 c.p.m.) of iodinated anti-HA IgG (radiolabelled using iodogen<sup>51</sup>) were added to the wells in wash buffer. After 2 h at 4 °C the excess antibody was aspirated and the plates washed as before. The wells were cut out from the microtitre plate and the <sup>125</sup>I bound to each well measured in a  $\gamma$ -counter. The number of HA molecules per cell was obtained by correction of the results of the radioimmunoassay for the fraction of cells infected (SVEHA3/CV1, 90%; 'flu/CV1, 30%; SVLHA8/COS-1, 5%; 'flu/COS-1, 20%), estimated as described in the text.

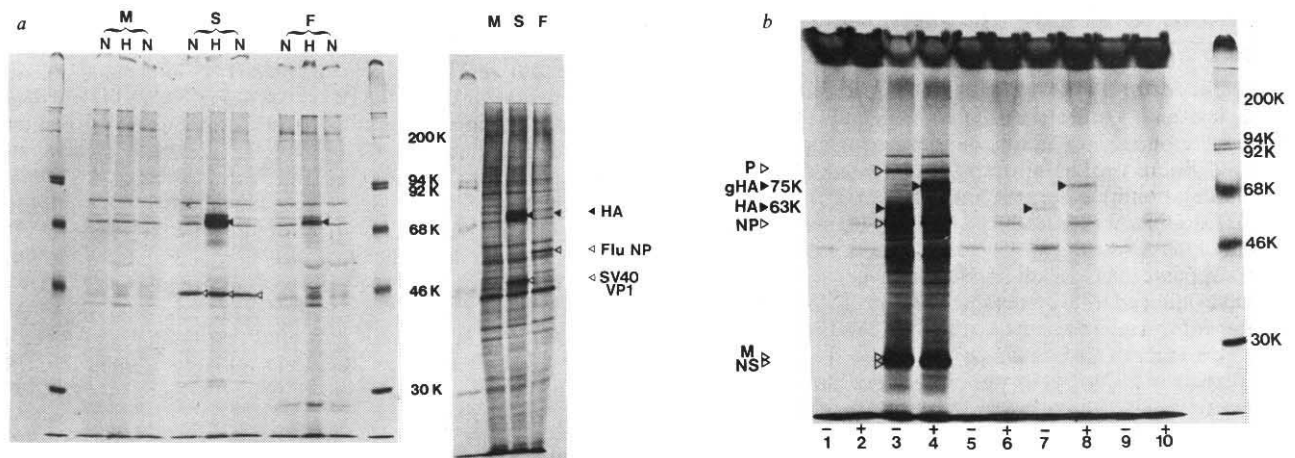
Extracts obtained from monolayers radiolabelled with <sup>35</sup>S-methionine between 46 and 48 h after infection with SVEHA3 were immunoprecipitated and analysed by SDS-polyacrylamide gel electrophoresis (PAGE). Figure 3a shows that these cell extracts contained a protein which was specifically precipitated by a monospecific anti-HA antiserum and which is indistinguishable in size ( $M_r = 75,000$ )<sup>26</sup> from authentic glycosylated HA precipitated from extracts of CV-1 cells infected for 10 h with the Japan strain of influenza virus. The HA species from SVEHA3-infected cells could be seen as a major band when the proteins in the labelled cell extract were separated by SDS-PAGE without prior precipitation. The results also indicate that the HAs synthesized in CV-1 cells infected by either SVEHA3 or influenza virus do not undergo activation cleavage<sup>27,55</sup> into HA<sub>1</sub> and HA<sub>2</sub> polypeptides. Figure 3b compares the products from cell-free translation of total poly(A)<sup>+</sup> RNA extracted from authentic influenza virus-infected cells and of SVEHA3 HA-specific RNA selected from a preparation of total, vector-infected cell RNA by hybridization to filters containing immobilized HA cDNA. Because the selection procedure is inefficient, it was not possible to compare the quantities of HA-specific mRNAs in the two preparations. However, cell-free translation showed that mRNA isolated from SVEHA3-infected cells directed the synthesis of a protein ( $M_r = 63,000$ ) which was the same size as the non-glycosylated form of authentic HA<sup>26</sup>. When the translation was carried out in the presence of dog pancreas membranes, the apparent molecular weight of both HA products increased to that of the *in vitro* glycosylated form of HA (75,000)<sup>26</sup>. Thus the *in vivo* and two *in vitro* forms of HA coded by SVEHA3 are indistinguishable from the corresponding forms coded by influenza virus itself.

The cellular location of the HA synthesized in SVEHA3-infected CV-1 cells was analysed by indirect immunofluorescent staining of cytoplasmic or surface antigens. Figure 4 shows that SVEHA3-infected cells showed bright cytoplasmic fluorescence, with an area, which is probably the Golgi apparatus, staining particularly brightly. CV-1 cells infected with influenza virus had a similar but slightly less bright pattern of immunofluorescence while mock-infected cells showed no fluorescence. Staining of SVEHA3 or influenza virus-infected cells fixed with formaldehyde showed more uniform but dimmer fluorescence over the entire cell surface.

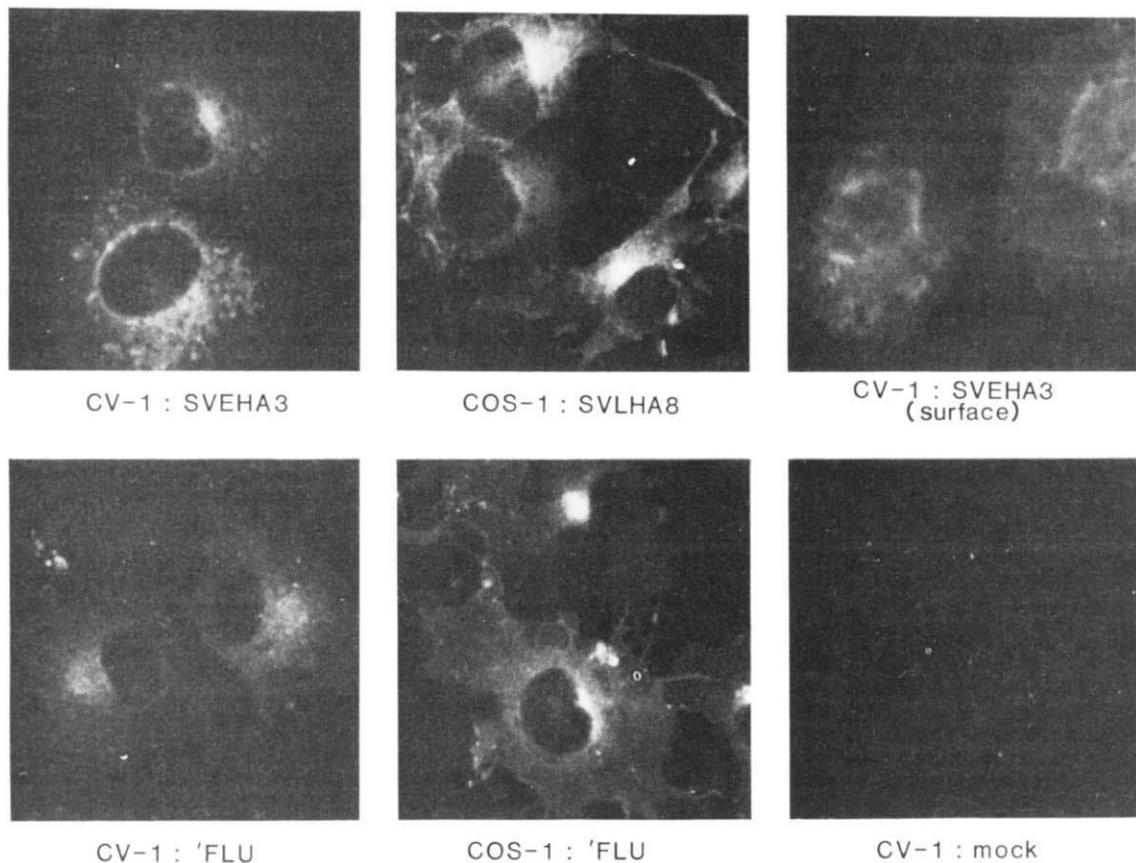
To confirm that HA is expressed at the surface of SVEHA3-infected cells two other types of experiment were performed: radioimmuno assay of intact cells and cell-surface haemagglutination of guinea pig erythrocytes (Fig. 5). By these tests the SVEHA3-infected cells are shown to display HA in a biologically active form in copious quantities on the cell surface. The only feature which distinguished these infected cells from influenza virus-infected cells was that erythrocytes which had been adsorbed to influenza-infected cells at 4 °C were eluted when the temperature was raised to 37 °C. Erythrocytes adsorbed to SVEHA3-infected cells remained attached after the temperature shift. This observation can be explained by the fact that influenza virus-coded neuraminidase, which causes red cells to be released from the surface of influenza virus-infected cells, is absent from cells infected with the late replacement vector SVEHA3. Taken together, these results show that cells infected with this vector synthesize large quantities of a HA which is indistinguishable from influenza virus HA by size, cellular location or ability to haemagglutinate erythrocytes.

When cells are infected with influenza virus, HA is budded from the cell surface as part of the virion and can be measured in the medium by assaying haemagglutinating activity<sup>28</sup>. Ten hours after infection by influenza virus, the 5 ml of medium covering the CV-1 cells contained a total of 2,560 HA units. By contrast, no haemagglutinating activity could be detected in the medium covering CV-1 cells 48 h after infection with SVEHA3. Thus the HA synthesized in SVEHA3-infected cells (which could be detected in cell extracts at a level of  $3.3 \times 10^4$  HA units per  $10^6$  cells) was not shed into the medium.





**Fig. 3** Comparison of HAs produced in SVHA3- or influenza virus-infected cells. *a*, Analysis by immunoprecipitation of HA from  $^{35}\text{S}$ -methionine-labelled cell extracts. CV-1 cell monolayers which had been infected with SVEHA3 or mock-infected (see Fig. 2 legend) were labelled 46–48 h post-infection with  $^{35}\text{S}$ -methionine (1 mCi per 90 mm dish, 1,000–3,000 Ci mmol $^{-1}$ ; Radiochemical Centre) in 1 ml E4 supplemented with 2% dialysed calf serum but lacking methionine. Similarly, influenza virus-infected monolayers were labelled 10–12 h post-infection. In this experiment, ~90% of the CV-1 cells treated with SVEHA3 became productively infected whereas ~20% of the cells treated with influenza virus were infected. The cell monolayers were then washed once with cold Tris-buffered saline and lysed at 0 °C for 20 min in 1.0 ml of lysis buffer (Fig. 2 legend). The cell debris was removed by centrifugation and the lysates used immediately for immunoprecipitation using rabbit anti-HA antiserum (H) or preimmune serum (N) as described previously<sup>48</sup>. The precipitated polypeptides (left panel) together with 1- $\mu\text{l}$  samples of non-precipitated cell extracts (right panel) were separated by SDS-PAGE and autoradiographed<sup>48</sup>. M, mock infected; S, SVEHA3 infected; F, influenza virus infected.  $\blacktriangleleft$  Indicates position of HA polypeptides,  $\blacktriangleright$  indicates positions of other viral (SV40 or influenza) polypeptides. *b*, *In vitro* translation of mRNA from SVEHA3- and influenza virus-infected CV-1 cells. Monolayers of CV-1 cells were infected with SVEHA3, or mock infected for 48 h, or infected with influenza virus for 10 h as described above (Fig. 2). Extraction of mRNA was performed as described by Favaloro *et al.*<sup>52</sup>. HA-specific mRNA was purified from total cytoplasmic RNA by hybridization to HA cDNA (pJHA)<sup>8</sup> covalently linked to amino phenylthioether paper (B. Seed, personal communication). *In vitro* translation was performed as described elsewhere<sup>26</sup> in the absence (–) or presence (+) of dog pancreas membranes<sup>26</sup>. Tracks 1, 2, 9 and 10, no added RNA; tracks 3 and 4, total cytoplasmic RNA from influenza virus-infected CV-1 cells; tracks 5 and 6, RNA eluted after hybridization to immobilized HA cDNA of cytoplasmic RNA from mock-infected cells; tracks 7 and 8, RNA eluted after hybridization to immobilized HA cDNA of cytoplasmic RNA from SVEHA3-infected cells. P, polymerase; NP, nucleocapsid protein; M, matrix; NS, non-structural protein.



**Fig. 4** Immunofluorescent antibody staining of HA in infected cells. Monolayers of CV-1 or COS-1 cells were infected with the recombinant vectors or mock-infected for 48 h, or infected with influenza virus for 10 h as described in Fig. 2 legend. For analysis of intracellular HA, cells growing on glass slides were fixed using ice-cold MeOH/acetone (1:1) for 10 min and extensively washed with PBS-A containing 0.5% BSA. For analysis of surface HA, the cells were fixed with formalin (10% solution in H $_2$ O) for 15 min before washing as above. Indirect immunofluorescent staining using rabbit anti-HA antiserum or preimmune rabbit serum followed by goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Cappel) was carried out using procedures described previously<sup>30</sup>.

## Expression of HA by the early replacement vector SVLHA8 in COS-1 cells

Because the vector SVLHA8 lacks the gene coding for large T antigen, it cannot replicate in simian cells unless functional T antigen is supplied. This can be done by using as a permissive host the COS-1 line of SV40-transformed monkey cells, which endogenously expresses large T antigen<sup>29</sup>.

SVLHA8 DNA was introduced without helper into COS-1 cells using DEAE-dextran<sup>24</sup>. The virus produced by these cells was serially passaged twice on fresh monolayers to obtain a virus stock which induced total cytopathic effect in COS-1 cells within 72 h after infection. The same virus stock produced no visible cytopathic effect in CV-1 cells.

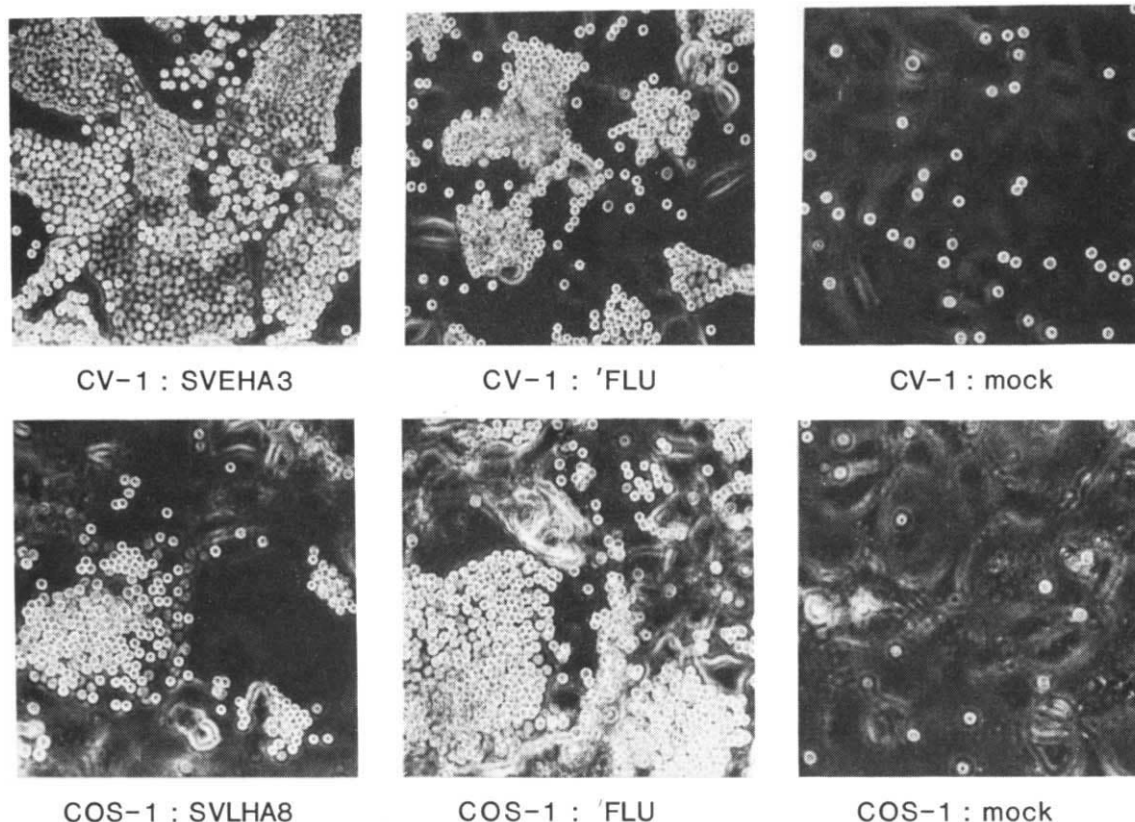
Monolayers of COS-1 cells were infected with this stock and at various times later the cells were analysed for the presence of HA by radioimmune assays, indirect immunofluorescence and haemabsorption as described above. Much less HA was detected by radioimmune assay of SVLHA8-infected COS-1 cell extracts (Fig. 2) than was detected in CV-1 cells infected with the late replacement vector SVEHA3. Indirect immunofluorescence and erythrocyte binding indicated that only a small proportion (~5%) of the cells were synthesizing HA (Figs 4 and 5). Immunofluorescence of fixed cells using antisera directed against SV40 VP1 and SV40 large T antigen<sup>30</sup> showed that a similar small percentage of cells were synthesizing SV40 V antigen, while all the cells were positive for large T antigen albeit with differing intensities (results not shown). The reason for the low rate of productive infection of the COS-1 cells is unknown. However, the fact that COS-1 cells infected with the late replacement vector SVEHA3 plus helper *dl1055* synthesized levels of HA similar to these in SVEHA3-infected CV-1 cells (results not shown) indicates that COS-1 cells are not inherently less receptive than CV-1 cells to infection by these SV40 vectors. This result suggests that at any one time, only a few COS-1 cells contain sufficient large T antigen to support efficient replication of SVLHA8 DNA.

Despite this low infection rate, the infected COS-1 cells synthesized HA which (1) exhibited the same pattern of immunofluorescence (Fig. 4) as SVEHA3-infected CV-1 cells; (2) was transported to the cell surface (Fig. 5); and (3) displayed haemagglutinating activity (Fig. 5). Correction of the results of the radioimmune assay (Fig. 2) for the fraction of cells infected (~5% of COS-1 cells compared with ~90% of CV-1 cells) indicated that COS-1 cells infected with SVLHA8 synthesized 10 times less HA than CV-1 cells infected with SVEHA3. This may reflect the less efficient expression of HA from the SV40 early promoter perhaps because of autoregulation by large T antigen<sup>31</sup> or because of gene dosage, as analysis of Hirt extracts<sup>32</sup> of infected cells indicated that the SVLHA8 recombinant genome was replicated to lower copy number in COS-1 cells than was SVEHA3 (plus *dl1055*) in either CV-1 or COS-1 cells (results not shown).

## Discussion

To our knowledge, influenza HA is the first integral membrane protein to be expressed in eukaryotic cells from recombinant DNA vectors. The protein appears normal in all respects; it is glycosylated and is displayed on the cell surface in an antigenic and biologically active form. Thus a protein which is naturally coded by a negative-stranded RNA genome can be expressed in copious amounts when DNA copies of its coding sequences are harnessed to strong papavovirus promoters. Furthermore no intervention by other influenza viral products is necessary to cause HA to accumulate on the cell surface. Taken together, these results provide evidence that the biosynthesis, transport, glycosylation and cellular localization of the viral protein are determined by host cell functions. Whether HA needs to interact with other virus-coded proteins (such as matrix) in order to bud from the cell surface remains to be established.

The amount of HA present in cells infected by the late SV40 replacement vector reaches levels which are never attained even during productive infection of these cells by the Japan influenza virus itself. From the strength of the signal obtained by Northern



**Fig. 5** Adsorption of erythrocytes to cell monolayers. CV-1 or COS-1 cells were infected with the recombinant vectors or mock-infected for 48 h, or infected with influenza virus for 10 h as described in Fig. 2 legend. The monolayers were washed twice with PBS and then 2 ml of a 0.5% solution of guinea pig erythrocytes in PBS was added. After 15 min at 4 °C, the monolayers were washed four times with PBS and photographed.



blotting (unpublished results) it was concluded that CV-1 cells infected with SVEHA3 contain about five times more HA mRNA than do cells infected with influenza virus. This excessive quantity of HA reflects in part the fact that cells infected with the recombinant vectors accumulate HA during the virus growth cycle, whereas cells infected with influenza virus shed HA as a normal part of the process of budding progeny virions. However, it also reflects the efficiency with which the HA gene is expressed from a recombinant DNA virus which replicates to high copy number.

Influenza HA therefore joins a number of other foreign genes which have been inserted into late replacement vectors of SV40 and expressed in simian cells<sup>33-36</sup>. Although it is difficult to obtain accurate estimates from the published data, it is clear that the level of expression of these genes is highly variable. Whereas cells infected with a late replacement vector carrying a cDNA copy of the rat preproinsulin gene synthesized  $\sim 10^6$  copies of proinsulin per cell<sup>36</sup>, those infected with vectors carrying cDNA or genomic copies of  $\beta$ -globin DNA synthesized  $>10^7$  copies per cell<sup>33,34</sup>. In only one system, however, is the foreign gene expressed in amounts comparable with influenza HA. Mulligan *et al.*<sup>33</sup> report that simian cells infected with SVGT-Ra $\beta$ G, which carries the cDNA sequences for rabbit  $\beta$ -globin in place of the gene coding for the SV40 capsid protein VP1, synthesize rabbit  $\beta$ -globin polypeptide in quantities nearly equivalent to the amount of VP1 produced by the helper virus. In our system influenza HA can be seen as a major band in extracts of radiolabelled vector-infected cells. We estimate that

about 5–10 times more HA than VP1 is produced in cells containing approximately equal numbers of helper and vector genomes.

The late replacement vector contains none of the splice donor or acceptor sites used in the synthesis of late SV40 mRNAs. It therefore seems likely that HA, like several other viral and cellular proteins<sup>37-41</sup>, is efficiently translated from an unspliced mRNA. A study of the structure of the HA mRNAs synthesized by SVEHA3 and SVLHA8 will be published elsewhere.

These experiments open the way to use site-directed mutagenesis to dissect those parts of the molecule important in its structure, function and biosynthesis and which have hitherto been inaccessible to genetic analysis.

Furthermore, it is now feasible to express hybrid proteins at the cell surface by inserting foreign nucleotide sequences into the vectors between the HA sequences which code for the hydrophobic signal and the membrane anchoring peptide. Modification of these vectors by removing the anchoring sequences may allow proteins which are normally found only in intracellular locations or attached to the membrane to be secreted from the host cells.

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# Insertion of DNA activates the cryptic *bgl* operon in *E. coli* K12

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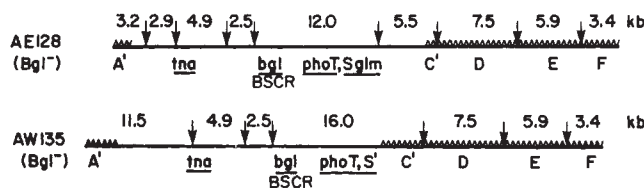
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*Spontaneous mutations which activate the cryptic bgl operon of Escherichia coli K12 are caused by insertion of DNA at a site, bglR, within the operon. Two insertion elements, IS1 and IS5, have been observed to effect this activation. Once the activating insertion has occurred the operon is inducible by  $\beta$ -glucosides in a cyclic AMP-dependent manner.*

TRANSPOSABLE DNA elements in bacteria are known to be involved in site-specific recombination events such as inversion, deletion and transposition<sup>1</sup>. The insertion of such elements into a gene usually results in inactivation of that gene and of distal genes in the same transcriptional unit. However, the insertion of

three transposable elements, IS2, TnA and Tn5 (refs 2, 3 and 4 respectively), can sometimes lead to activation of a downstream gene or operon due to the presence of promoters within these transposons. Expression from these promoters is unregulated and requires a unique orientation or location of the transposon relative to the gene being expressed. We demonstrate here that activation of the cryptic *bgl* operon in *E. coli* K12 occurs

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**Fig. 1**  $\lambda$  specialized transducing phages carrying the *bgl* operon. The specialized transducing phage  $\lambda$ AE128 was isolated by a two-step procedure starting with a lysogen (JF225) containing  $\lambda$ Y199 (Acl857 xis(Am) S7b515b519) integrated at a secondary attachment site in the *phoS* gene of *E. coli* K12. Induction of JF225 gave rise to a specialized transducing phage carrying the region from *phoS* through *tnaA*, identified by its ability to transduce a *tnaA*<sup>-</sup> cell to *tnaA*<sup>+</sup>. A lysogen (JF369) carrying this transducing phage, which was integrated by homology in the *tnaA* region of the chromosome, was induced giving rise to specialized transducing phages carrying the chromosomal region from *tnaA* to *glmS*.  $\lambda$ AE128 is one such phage, identified by its ability to transduce a *glmS*<sup>-</sup> cell to *glmS*<sup>+</sup> (ability to synthesize glucosamine).  $\lambda$ AE128 is a defective phage which carries the chromosomal alleles *glmS*<sup>+</sup>, *tnaA*<sup>+</sup> and *bglR*<sup>0</sup>.  $\lambda$ AW135 was isolated by inducing a *phoS*:: $\lambda$ Y199 lysogen (AE120), infecting a *tnaA*<sup>-</sup> strain with the resulting lysate and selecting *tnaA*<sup>+</sup> transductants. One of these transductants, AW135, was used as the source of the *tnaA*<sup>+</sup> *bglR*<sup>0</sup> transducing phage  $\lambda$ AW135.  $\lambda$ AE128 has an *EcoRI* restriction site in the segment of bacterial DNA between *A'* and *tna* which is absent in the equivalent segment of  $\lambda$ AW135 bacterial DNA. This restriction site is characteristic of the particular strain from which  $\lambda$ AE128 was derived. — Indicates bacterial DNA,  $\lambda$  indicates  $\lambda$  DNA,  $\downarrow$  indicates the *EcoRI* restriction sites.

spontaneously by insertion of either IS1 or IS5 into the operon. In contrast to the above examples of activation, expression of this 'insertion-activated' operon is subject to substrate-specific regulation. The mechanism which we describe for activation of the *bgl* operon represents a novel system of genetic regulation by transposition of insertion sequences.

The *bgl* operon, which is located near minute 83 on the *E. coli* K12 genetic map, contains three structural genes required for the catabolism of aromatic  $\beta$ -glucosides, such as salicin and arbutin<sup>5</sup>. The operon is considered to be cryptic because these genes cannot be expressed, or induced, in wild-type *E. coli* K12. Wild-type bacteria are thus unable to ferment salicin (Sal<sup>-</sup>) or arbutin (Arb<sup>-</sup>). However, Bgl<sup>+</sup> (Sal<sup>+</sup>, Arb<sup>+</sup>) mutants can arise spontaneously, and in these mutants the operon is inducible by various  $\beta$ -glucosides. The genes of the *bgl* operon are *bglB*, which encodes a hydrolytic enzyme, phospho- $\beta$ -glucosidase B; *bglS*, whose product is a regulator of the *bgl* operon; and *bglC*, which encodes a specific transport activity<sup>6</sup>. In addition to these three structural genes there exists a site, *bglR*, which is required for their expression, and where the spontaneous mutations allowing expression of the *bgl* operon occur<sup>5</sup>. We have termed the mutations giving rise to the Bgl<sup>+</sup> phenotype, *bglR*, and have designated the wild-type allele *bglR*<sup>0</sup>, because it gives a Bgl<sup>-</sup> phenotype. (The nomenclature used here for the *bglR* site differs from that used by Prasad and Schaefer<sup>5</sup> in the following ways. We have used *bglR*<sup>0</sup> in place of *bglR*<sup>+</sup> to indicate the cryptic nature of the wild-type site, and *bglR* instead of *bglR*<sup>-</sup> to indicate the mutant site which allows expression of the *bgl* operon.)

Figure 1 shows the organization of the genes of the *bgl* operon. This gene order, which differs from that originally proposed by Prasad and Schaefer<sup>5</sup>, was determined by a physical and genetic analysis of the *bgl* operon cloned in plasmid pBR322 (in preparation). Merodiploid analysis has shown that *bglR* is dominant to *bglR*<sup>0</sup> and that expression of genes *B*, *S* and *C* requires a *bglR* allele *cis* to the structural genes<sup>5</sup>. The *S* product is *trans*-acting and according to the model proposed by Prasad and Schaefer<sup>5</sup> is a positive regulator required for expression of genes *bglB* and *bglC*. The location of the *bglR* mutation and its *cis* action on the expression of the operon strongly suggest that transcription of the *bgl* operon is initiated near *bglR*.

The requirements for the *bglR* mutation, with its *cis*-acting effects, are so specific that it would be expected to occur only at very low frequency. In fact, it occurs at frequencies as high as 10<sup>-5</sup> in some *E. coli* K12 strains, suggesting that a special mechanism may exist for the spontaneous *bglR*<sup>0</sup> to *bglR* muta-

tion. We considered that DNA rearrangements might be involved and tested this hypothesis by comparing the DNA structure of operons containing *bglR*<sup>0</sup> and *bglR* alleles.

## Specialized transducing phages carry the *bgl* operon

To examine the structure of the *bgl* operon, we isolated the  $\lambda$ Bgl<sup>-</sup> specialized transducing phages shown in Fig. 1. These phages were generated by induction of lysogens containing  $\lambda$  prophages in secondary attachment sites near the *bgl* operon. Two Bgl<sup>-</sup> phages were used in this work;  $\lambda$ AE128, which carries a segment of bacterial chromosome from *tnaA* through *glmS*, and  $\lambda$ AW135, which carries DNA from *tnaA* to *phoS*. The transducing phages  $\lambda$ AE129 and  $\lambda$ AE130 are independent spontaneous Bgl<sup>+</sup> derivatives of  $\lambda$ AE128, and  $\lambda$ AW138 is a spontaneous Bgl<sup>+</sup> derivative of  $\lambda$ AW135.

We assayed phospho- $\beta$ -glucosidase B activity in cells lysogenic for the series of Bgl<sup>-</sup> and Bgl<sup>+</sup> transducing phages to show that the phage-carried operon is subject to the same regulation as the operon in its normal chromosomal location. The results (see Table 1) indicate that the Bgl<sup>-</sup> lysogens AE128 and AW135 showed no phospho- $\beta$ -glucosidase B activity either in the absence or presence of inducer. In contrast, the Bgl<sup>+</sup> lysogens AE129, AE130 and AW138 did show phospho- $\beta$ -glucosidase B activity, but only when inducer was present. The Bgl<sup>-</sup> and Bgl<sup>+</sup> lysogens are therefore regulated in the same way as the non-lysogenic haploid Bgl<sup>-</sup> strain RVA and its Bgl<sup>+</sup> derivative. The Bgl<sup>-</sup> lysogens exhibit a range of enzyme levels which correlates with the intensity of their colour development on MacConkey salicin indicator plates.

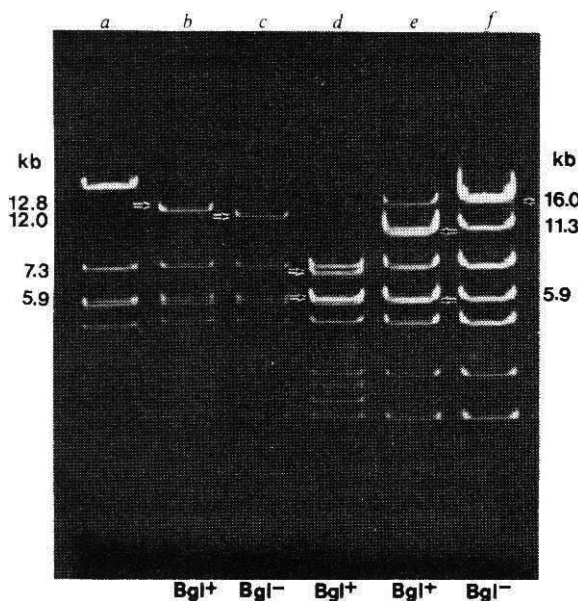
After activation by a *bglR* mutation, expression of the *bgl* operon, in addition to being inducible by  $\beta$ -glucosides, is dependent on cyclic AMP. We showed this by constructing a *bglR* strain ( $\Delta$ *cya*) which carries a deletion of the gene for adenylate cyclase, *cya*. This strain is Lac<sup>-</sup> Mal<sup>-</sup> Bgl<sup>-</sup> on indicator plates, unlike its isogenic *cya*<sup>+</sup> parent which is Lac<sup>+</sup> Mal<sup>+</sup> Bgl<sup>+</sup>. The defect in the  $\Delta$ *cya* strain can be corrected by the addition of

**Table 1** Specific activity of phospho- $\beta$ -glucosidase B enzyme in Bgl<sup>-</sup> and Bgl<sup>+</sup> strains

| Strain                  | <i>bgl</i> genotype                                           | Phenotype        | Phospho- $\beta$ -glucosidase B activity |              | Induced activity |
|-------------------------|---------------------------------------------------------------|------------------|------------------------------------------|--------------|------------------|
|                         |                                                               |                  | Without inducer                          | With inducer |                  |
| RVA                     | <i>bglR</i> <sup>0</sup>                                      | Bgl <sup>-</sup> | 0.000                                    | 0.007        | 0.007            |
| RVA (Bgl <sup>+</sup> ) | <i>bglR</i>                                                   | Bgl <sup>+</sup> | -0.013                                   | 0.66         | 0.673            |
| AW135                   | $\lambda$ <i>bglR</i> <sup>0</sup> / <i>bglR</i> <sup>0</sup> | Bgl <sup>-</sup> | 0.02                                     | 0.035        | 0.015            |
| AW138                   | $\lambda$ <i>bglR</i> / <i>bglR</i> <sup>0</sup>              | Bgl <sup>+</sup> | 0.068                                    | 0.458        | 0.390            |
| AE128                   | $\lambda$ <i>bglR</i> <sup>0</sup> / <i>bglR</i> <sup>0</sup> | Bgl <sup>-</sup> | 0.013                                    | 0.014        | 0.001            |
| AE129                   | $\lambda$ <i>bglR</i> / <i>bglR</i> <sup>0</sup>              | Bgl <sup>+</sup> | 0.034                                    | 0.222        | 0.188            |
| AE130                   | $\lambda$ <i>bglR</i> / <i>bglR</i> <sup>0</sup>              | Bgl <sup>+</sup> | -0.007                                   | 0.013        | 0.020            |
| AE141                   | $\lambda$ <i>bglR</i> / <i>bglR</i> <sup>0</sup>              | Bgl <sup>+</sup> | 0.017                                    | 0.039        | 0.022            |
| KO618                   | <i>bglR</i>                                                   | Bgl <sup>+</sup> | -0.047                                   | 0.131        | 0.178            |

Cells were grown to mid-log phase in M9 medium containing succinate (0.4%) as a carbon source, casamino acids (0.2%), thiamine (10  $\mu$ g ml<sup>-1</sup>), tryptophan (50  $\mu$ g ml<sup>-1</sup>) and with or without  $\beta$ -methylglucoside (5  $\times$  10<sup>-3</sup> M) as inducer. Cells were washed with 0.8% saline and resuspended in saline to one-tenth of the original volume. Phospho- $\beta$ -glucosidase B activity was assayed as follows<sup>20</sup>. 0.1 ml of 0.14 M salicin and 0.1 ml of cell suspension were mixed and incubated for 30 min at 37 °C. The reaction was stopped by addition of 0.5 ml of 2M Na<sub>2</sub>CO<sub>3</sub>. To each sample, at room temperature, 0.5 ml of a 0.6% solution of 4-amino-antipyrine was added, followed 15 min later by 0.5 ml of 4% K<sub>3</sub>Fe(CN)<sub>6</sub>. After 5 min at room temperature the optical density (A) at 509 nm was measured. This measurement was corrected for the contribution made by turbidity. Specific activity was determined by dividing the corrected A<sub>509</sub> by the optical density of the cell culture measured at 600 nm. The values in the table are given relative to phospho- $\beta$ -glucosidase B activity in the wild-type haploid strain (RVA) when grown in the absence of inducer. The control level of activity for lysogens AE129, AE130 and AE141 is that given by the parent Bgl<sup>-</sup> lysogen AE128. The Bgl<sup>-</sup> lysogen, AW135, is the analogous control for AW138. AW135, AW138, AE128, AE129 and AE130 are lysogens carrying the  $\lambda$ Bgl<sup>-</sup> and  $\lambda$ Bgl<sup>+</sup> specialized transducing phages as described in the text. AE141 is lysogenic for  $\lambda$ Bgl<sup>+</sup> phage derived by mutagenesis of the  $\lambda$ Bgl<sup>-</sup> phage present in AE128. KO618 is a Bgl<sup>+</sup> strain derived by mutagenesis of the Bgl<sup>-</sup> haploid strain RVA. Mutagenesis was carried out by placing filter disks, spotted with either ethylmethanesulphonate or nitroguanidine, on MacConkey salicin agar which had been spread with the Bgl<sup>-</sup> strain. Red papillae (Bgl<sup>+</sup> mutants) which appeared were isolated and purified for study.





**Fig. 2** *EcoRI* restriction fragment patterns of *Bgl*<sup>-</sup> and *Bgl*<sup>+</sup> specialized transducing phages. Phages were obtained by heat induction of the appropriate lysogen and purified in CsCl gradients. DNA was extracted from the phages with phenol and samples (0.75 µg) were digested with *EcoRI* restriction endonuclease. DNA fragments were separated by electrophoresis on 0.9% agarose using a buffer containing 50 mM sodium acetate, 10 mM EDTA and 0.4 M Tris pH 7.9 for 2 h at 120 V. An *EcoRI* digest of  $\lambda$ cl857 S7(Am) DNA was used as a size standard<sup>21</sup>. a,  $\lambda$ cl857S7; b,  $\lambda$ AE128; c,  $\lambda$ AE129; d,  $\lambda$ AE129; e,  $\lambda$ AW138; f,  $\lambda$ AW135.

1 mM cyclic AMP to the indicator plates, thereby restoring the Lac<sup>+</sup> Mal<sup>+</sup> *Bgl*<sup>+</sup> phenotype.

### Restriction enzyme analysis of *Bgl*<sup>-</sup> and *Bgl*<sup>+</sup> transducing phages

Figure 2 shows the *EcoRI* restriction fragment patterns of the  $\lambda$  specialized transducing phages  $\lambda$ AE128,  $\lambda$ AW135 and their respective isogenic *Bgl*<sup>+</sup> derivatives. Cloning evidence (A.E.R., unpublished observations) has revealed that the largest *EcoRI* fragment (12.0 kilobases [kb]) of  $\lambda$ AE128 contains the entire *bgl* operon and Southern hybridization has shown that the fragment consists only of chromosomal DNA. In the case of  $\lambda$ AW135, the largest fragment (16.0 kb) contains almost all of the *EcoRI* fragment carrying the *bgl* operon and part of  $\lambda$ *EcoRI* fragment C (see Fig. 1). The order of restriction fragments in the transducing phages is based on mapping data of the *oriC* region of the *E. coli* chromosome<sup>7</sup>.

The *bglR*<sup>0</sup>-containing *EcoRI* fragments of  $\lambda$ AE128 and  $\lambda$ AW135 are altered by spontaneous mutation to *bglR*. The uppermost fragment of  $\lambda$ AE130 (Fig. 2) is slightly larger than that of the parent *bglR*<sup>0</sup> phage  $\lambda$ AE128, indicating that additional DNA is present in the *bglR* derivative.  $\lambda$ AE129 and  $\lambda$ AW138 lack the uppermost fragment present in their respective parental phages but contain two new fragments, 7.3 kb and 5.9 kb in the case of  $\lambda$ AE129, and 11.3 kb and 5.9 kb in the case of  $\lambda$ AW138. In each case the 5.9-kb fragment coincides with *EcoRI* fragment E of phage  $\lambda$ , as the band has the intensity of a doublet. These results with *EcoRI* and results from analyses using *HindIII* and *PstI* (data not shown) indicate that mutation from *Bgl*<sup>-</sup> to *Bgl*<sup>+</sup> is correlated in each case with insertion of DNA.

To measure more accurately the sizes of the insertions, we compared the *HincII* + *HindIII* restriction patterns of the *Bgl*<sup>-</sup> and *Bgl*<sup>+</sup> transducing phages. As shown in Fig. 3, a 910-base pair (bp) fragment which is present in both *Bgl*<sup>-</sup> transducing phages is missing in all the *Bgl*<sup>+</sup> derivatives, indicating that the insertion affecting the *bglR* locus has occurred somewhere within the same 910-bp segment of DNA in each spontaneous *Bgl*<sup>+</sup> mutant. In  $\lambda$ AE130 a new, larger fragment forms a doublet with the 1.70-kb fragment already present in the parent phage. This fragment was clearly resolved in a polyacrylamide-agarose gel

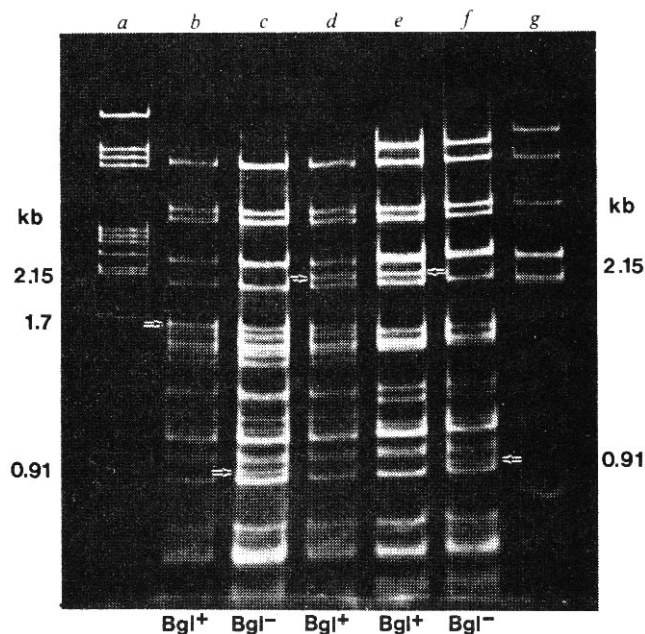
system (result not shown). Thus the insertion in  $\lambda$ AE130 is 790 bp. A new fragment of 2.15 kb is seen in both  $\lambda$ AE129 and  $\lambda$ AW138, indicating an insertion of 1,240 bp.

Thus two different DNA insertion events are correlated with the spontaneous mutations from *Bgl*<sup>-</sup> to *Bgl*<sup>+</sup>. Eight independent spontaneous *Bgl*<sup>+</sup> mutations have been analysed, using *bgl* phages, as described above; four were found to be due to the smaller insertion (790 bp), the other four to the larger insertion (1,240 bp). Each insertion was found to occur in *Bgl*<sup>+</sup> derivatives of either of the *Bgl*<sup>-</sup> parent phages,  $\lambda$ AE128 and  $\lambda$ AW135. Because the wild-type *bgl* operon present on  $\lambda$ AE128 originated from a different *E. coli* K12 strain line than that giving rise to  $\lambda$ AW135, we believe that activation of the *bgl* operon by insertion of DNA is a general phenomenon.

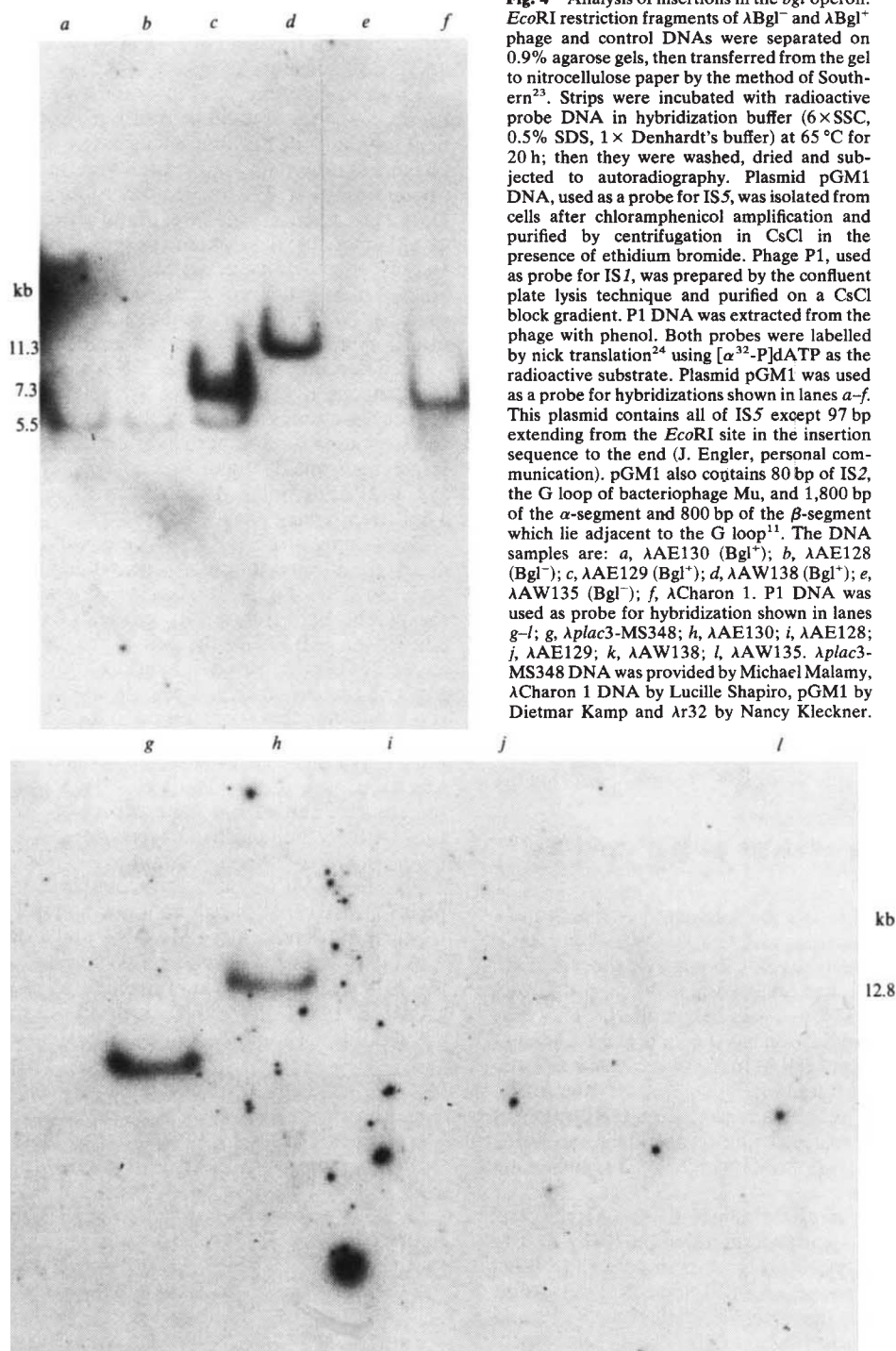
To determine whether the DNA insertions present in the spontaneous *Bgl*<sup>+</sup> derivatives are related to known insertion sequences, a series of DNA probes containing IS1, IS2, IS3 and IS5 were used for Southern hybridization analysis of restriction fragments from *Bgl*<sup>-</sup> and *Bgl*<sup>+</sup> phages. Only those containing IS1 and IS5 hybridized specifically to *bgl* operon DNA from  $\lambda$ *Bgl*<sup>+</sup> transducing phages.

Bacteriophage P1 DNA, which contains IS1 (ref. 8), hybridized specifically to the *bgl*-containing *EcoRI* fragment of  $\lambda$ AE130 DNA (Fig. 4). It failed to hybridize to DNA from either the parent *Bgl*<sup>-</sup> transducing phage,  $\lambda$ AE128, its other *Bgl*<sup>+</sup> derivative,  $\lambda$ AE129, or the pair of phages  $\lambda$ AW135 (*Bgl*<sup>-</sup>) and  $\lambda$ AW138 (*Bgl*<sup>+</sup>), but did hybridize with the *lac*-containing *EcoRI* fragment of  $\lambda$ plac3-MS348 which contains IS1<sup>9</sup>. These results indicate that the insertion in the DNA of the *Bgl*<sup>+</sup> phage  $\lambda$ AE130 is homologous to IS1. Similar results indicating that the insertion in  $\lambda$ AE130 is IS1 were obtained when  $\lambda$ plac3-MS348 was used as a probe. The size of the insertion in  $\lambda$ AE130 (determined above) and the presence of *PstI*, *PvuII* and *BalI* sites (A.E.R., unpublished observations) within the insertion<sup>10</sup> is consistent with this conclusion.

Plasmid pGM1 was used as a probe for IS5. This plasmid<sup>11</sup> is a pBR322 derivative which contains about 1,150 bp of IS5, a segment of bacteriophage Mu DNA and a small portion of IS2 (see Fig. 4 legend). Plasmid pGM1 hybridized with one of the two *bglR*-specific *EcoRI* subfragments of  $\lambda$ AE129 (7.3 kb) and  $\lambda$ AW138 (11.3 kb). It did not hybridize to the *bgl* fragment of



**Fig. 3** *HincII*-*HindIII* restriction fragment patterns of *Bgl*<sup>-</sup> and *Bgl*<sup>+</sup> specialized transducing phages. Phage DNA was digested with *HincII*-*HindIII* restriction endonuclease mixture (New England Biolabs). The DNA fragments were separated by electrophoresis on 1.4% agarose at 120 V for 2 h. The size standard is a *HpaI* digest of phage T7 DNA<sup>22</sup>. a, T7, *HpaI* digest; b,  $\lambda$ AE130; c,  $\lambda$ AE128; d,  $\lambda$ AE129; e,  $\lambda$ AW138; f,  $\lambda$ AW135; g,  $\lambda$ cl857S7, *HincII*-*HindIII* digest.



**Fig. 4** Analysis of insertions in the *bgl* operon. *Eco*RI restriction fragments of  $\lambda$ Bgl<sup>-</sup> and  $\lambda$ Bgl<sup>+</sup> phage and control DNAs were separated on 0.9% agarose gels, then transferred from the gel to nitrocellulose paper by the method of Southern<sup>23</sup>. Strips were incubated with radioactive probe DNA in hybridization buffer (6×SSC, 0.5% SDS, 1×Denhardt's buffer) at 65 °C for 20 h; then they were washed, dried and subjected to autoradiography. Plasmid pGM1 DNA, used as a probe for IS5, was isolated from cells after chloramphenicol amplification and purified by centrifugation in CsCl in the presence of ethidium bromide. Phage P1, used as probe for IS1, was prepared by the confluent plate lysis technique and purified on a CsCl block gradient. P1 DNA was extracted from the phage with phenol. Both probes were labelled by nick translation<sup>24</sup> using [ $\alpha^{32}$ -P]dATP as the radioactive substrate. Plasmid pGM1 was used as a probe for hybridizations shown in lanes a–f. This plasmid contains all of IS5 except 97 bp extending from the *Eco*RI site in the insertion sequence to the end (J. Engler, personal communication). pGM1 also contains 80 bp of IS2, the G loop of bacteriophage Mu, and 1,800 bp of the  $\alpha$ -segment and 800 bp of the  $\beta$ -segment which lie adjacent to the G loop<sup>11</sup>. The DNA samples are: a,  $\lambda$ AE130 (Bgl<sup>+</sup>); b,  $\lambda$ AE128 (Bgl<sup>-</sup>); c,  $\lambda$ AE129 (Bgl<sup>+</sup>); d,  $\lambda$ AW138 (Bgl<sup>+</sup>); e,  $\lambda$ AW135 (Bgl<sup>-</sup>); f,  $\lambda$ Charon 1. P1 DNA was used as probe for hybridization shown in lanes g–l; g,  $\lambda$ plac3-MS348; h,  $\lambda$ AE130; i,  $\lambda$ AE128; j,  $\lambda$ AE129; k,  $\lambda$ AW138; l,  $\lambda$ AW135.  $\lambda$ plac3-MS348 DNA was provided by Michael Malamy,  $\lambda$ Charon 1 DNA by Lucille Shapiro, pGM1 by Dietmar Kamp and  $\lambda$ r32 by Nancy Kleckner.

the parent Bgl<sup>-</sup> phages  $\lambda$ AE128 and  $\lambda$ AW135 or to that of the Bgl<sup>+</sup> phage  $\lambda$ AE130. pGM1 hybridized to  $\lambda$ Charon 1 DNA which contains only IS5 in common with the plasmid<sup>12</sup>. The *bglR*-specific fragments of  $\lambda$ AE129 and  $\lambda$ AW138 which hybridize to pGM1 did not hybridize with Mu DNA,  $\lambda$ r32 (IS2)<sup>13</sup> DNA or pBR322 DNA. Thus the insertion in the *bgl* region of  $\lambda$ AE129 and  $\lambda$ AW138 is homologous to IS5. The size of the DNA insertion in these two phages and the presence of an *Eco*RI site within the insertion (previous section) lead us to conclude that this insertion is IS5 in both cases. From this hybridization data and the restriction analysis of the phages we conclude that the orientation of IS5 in *bglR* is the same in both cases. Within the limits of resolution of the gel system used, the point of insertion is also the same, being 5.8 kb from the *Eco*RI site which is indicated at the immediate left end of the *bgl* operon in Fig. 1.

pGM1 also hybridized to another *Eco*RI fragment present in

the family of phages  $\lambda$ AE128 (Bgl<sup>-</sup>),  $\lambda$ AE129 (Bgl<sup>+</sup>) and  $\lambda$ AE130 (Bgl<sup>+</sup>). This 5.5-kb fragment lies in the *glmS-uncC,D* region of the chromosome<sup>7</sup> which is not present in  $\lambda$ AW135 or  $\lambda$ AW138. The presence of IS5 or a portion of IS5 in this 5.5-kb fragment is not specific to *bgl*, is well separated from the *bgl* operon and does not seem to be related to expression of the *bgl* operon.

### Mutagen-induced *bgl*<sup>+</sup> strains

The mutagens ethylmethanesulphonate (EMS) and nitrosoguanidine (NTG) increase the frequency with which Bgl<sup>+</sup> papillae appear in a lawn of a Bgl<sup>-</sup> strain on MacConkey salicin indicator plates. As mutagens are not known to increase the frequency of transposition of insertion sequences, we wished to determine whether mutagen-induced Bgl<sup>+</sup> mutations were due to insertions and whether they showed the same regulatory



properties and dominance relationships as spontaneous  $Bgl^+$  mutations. We therefore isolated EMS- and NTG-induced  $Bgl^+$  mutants in both a haploid ( $bglR^0$ ) and merodiploid ( $bglR^0/\lambda bglR^0$ ) background. Twelve  $Bgl^+$  mutants were isolated from the  $Bgl^-$  lysogen AE128. Two of the mutations which led to the  $Bgl^+$  phenotype were located on the specialized transducing phage genome. The *HincII*-*HindIII* and *EcoRI* restriction patterns of these phages were identical to those of the  $Bgl^-$  parent phage. Thus neither of these phages contained the *IS1* or *IS5* insertions seen in the spontaneous  $Bgl^+$  mutants. In 10 of the 12  $Bgl^+$  mutants isolated, the mutations were present on the chromosome and were dominant to  $bglR^0$  on the phages.

The haploid strain RVA ( $Bgl^-$ ) was mutagenized with EMS or NTG and  $Bgl^+$  mutants were selected. In 12 such mutants the *bgl* mutation was P1-cotransducible with *tnaA* at a frequency of 65–90%, which is characteristic of spontaneous *bglR* mutations. When a  $Bgl^-$  phage ( $\lambda$ AW135) was introduced into strains carrying these *bgl* mutations, the resulting lysogens showed a  $Bgl^+$  phenotype in all cases, indicating dominance of the mutagen-induced *bgl* mutations over the wild type.

The mutagen-induced *bgl* mutations thus appear very similar to spontaneous *bglR* mutations in that they show the same dominance properties and the operons which they activate are inducible by  $\beta$ -glucosides (Table 1). However they are distinctly different from the spontaneous mutations because they show no evidence of an insertion in the *bgl* region.

## Discussion

Spontaneous mutations which activate the cryptic *bgl* operon in *E. coli* K12 occur at a specific region within the *bglR* operon<sup>5</sup>. We have demonstrated here that these mutations are due to insertion of *IS1* or *IS5* into a specific region in the operon. Operon expression in the mutants is inducible, dependent on cyclic AMP and requires the presence of the insertion in *cis*. We have considered two different models to explain how insertion of *IS1* or *IS5* could effect the *cis* activation of this operon. In the first model, the *bgl* operon lacks a functional promoter for *bglC*, *bglS* and *bglB*; insertion of DNA into the *bglR* region would either activate a pre-existing promoter or provide or contribute to promoter structure. Because the properties of other insertion mutations produced by *IS1* and *IS5* indicate that these elements do not show promoter activity<sup>1</sup>, it seems unlikely that they could provide a complete promoter sequence for transcription of the *bgl* operon. In the second model, the operon contains an operator site which is disrupted by the insertion sequence. The existence of an operator sequence would imply the existence of a repressor of the operon. Such a repressor would be refractory to induction by  $\beta$ -glucosides. Defez and DeFelice<sup>14</sup> have recently described mutations at a locus, *bglY*, which are recessive and which lead to a  $Bgl^+$  phenotype. They suggested that *bglY*, which maps near *trp*, may code for a repressor of the *bgl* operon. However, in our strains, analysis of many spontaneous and mutagen-induced  $Bgl^+$  derivatives has failed to provide any evidence for such a repressor; all  $Bgl^+$  derivatives analysed contained dominant mutations located within the *bgl* operon. The  $Bgl^+$  phenotype conferred by *bglY* mutations may therefore be strain specific. Further work will be necessary to determine the role of the *bglY* product in *bgl* operon expression.

Analysis of two independent *IS5* insertions and one *IS1* insertion indicated that all three are located within 50–100 nucleotides of another. The insertions probably do not occur at

the same nucleotide because spontaneous  $Bgl^+$  mutants vary in their levels of operon expression. The small size of the insertion target and the relatively high frequency at which the spontaneous  $Bgl^-$  to  $Bgl^+$  mutation occurs leads us to conclude that *bglR* is a preferred site for insertion of both *IS1* and *IS5*.

All the spontaneous mutations that we have analysed have been due to insertion of DNA. However, the frequency of mutation to  $Bgl^+$  can be stimulated by treatment with chemical mutagens. These mutations have similar biological properties to the spontaneous mutations but do not seem to be due to insertion of DNA. Although it is possible that the mutagen-induced mutations activate the operon by a mechanism similar to that used by the insertions, this may not be the case.

Activation of gene expression by site-specific recombination can occur by inversion, as in phase variation in *Salmonella*<sup>15</sup>; by substitution, seen in control of mating type in yeast<sup>16</sup>; by deletion, as occurs during differentiation of immunoglobulin-producing cells in the mouse<sup>17</sup>; and by insertion, such as in the activation of a cellular *onc* gene in virus-induced lymphoid leukosis<sup>18</sup>. All these processes appear to be part of specific systems of biological control; however, there are many examples of alterations of gene expression by non-homologous recombination which do not appear to occur by specific regulatory mechanisms. Examples include inactivation of genes by insertion of transposable genetic elements and nonspecific activation of a gene by integration of an insertion sequence carrying a promoter (for example, *IS2*)<sup>2</sup>. The existence of a preferred site for integration of two *IS* sequences into the *bgl* operon, the activating effect of such an integration event and the subsequent dual regulation of the activated operon suggest that this activation may be part of a specific system of biological control which confers some evolutionary advantage on cells possessing it.

If this system is used to turn the *bgl* operon of *E. coli* on and off repeatedly, the reverse mutation, *bglR* to *bglR*<sup>0</sup>, must also occur and in fact it has been observed in a particular mutant background which has an increased frequency of precise excision of transposons such as *Tn5* and *Tn10* (J. Hopkins and M. Syvanen, personal communication). One possible reason why the type of physiological regulation seen in operons such as the lactose operon might not be adequate for the *bgl* operon is that many  $\beta$ -glucosides are produced in nature, some of which are toxic, for example, the cyanogenic  $\beta$ -glucosides<sup>19</sup>. Such toxic  $\beta$ -glucosides might be inducers of the *bgl* operon and inhibitory to cells which metabolize them, but not to  $Bgl^-$  cells. Mutation from *bglR* to *bglR*<sup>0</sup> would enable the cell to survive exposure to toxic  $\beta$ -glucosides while conserving the structural genes of the operon in a form which could be easily reactivated by a *bglR* mutation at a later time.

Activation of cryptic genes by insertion mutations may occur in other systems as well and may be a widespread method of control. This method of mutational genetic regulation may be a specific example of the general class of regulation by recombination.

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# LETTERS TO NATURE

## Daily and seasonal Viking observations of martian bore wave systems

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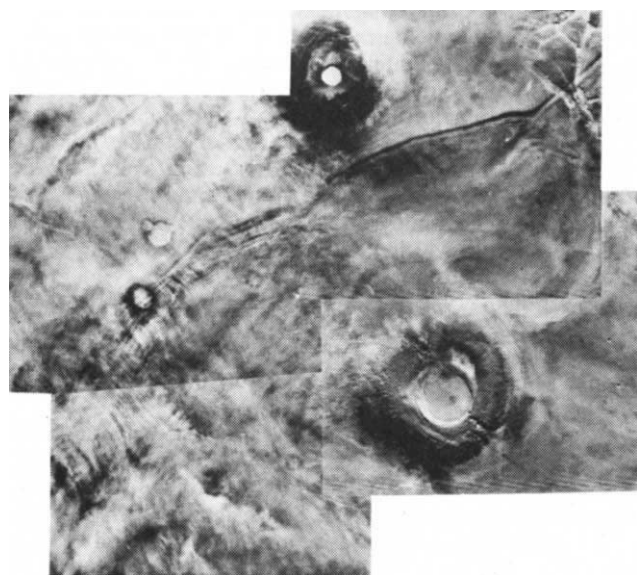
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A martian atmospheric phenomenon called a 'bore wave' has been observed by the Viking imaging system during late spring and early summer of two martian years, in the Tharsis Ridge region of the planet. Here we present the observational data and offer a tentative explanation for the occurrence of this feature, formed by airflow and which behaves like a thermally induced diurnal katabatic breeze.

The bore wave has been observed on at least one occasion during each of the three late springs and early summers in the northern hemisphere by the Viking orbiters. One of the 1976 observations of the Viking primary mission has been discussed by Briggs *et al.*<sup>1</sup>. Their Fig. 4 reveals an example of the bore wave obtained on orbit 62A. Table 1 gives information on the times relative to the annual and diurnal cycles of this phenomenon.



**Fig. 1** Mosaic of the region between Arsia Mons and Pavonis Mons obtained during the Viking Survey Mission at  $L_s = 100.5^\circ$ . The local time is 07.05, and it refers to the specific event shown. This system is used in all the images, and the pictures have been filtered to remove low-frequency albedo variations and thereby enhance details. This picture clearly shows the eastern branch over Noctis Labyrinthus and the associated kink at the Claritas Fossae Ridge. There is a slight indication of a second branch to the west of the ridge which is more prominent in other sequences (404S22–25). Top left ( $1.6^\circ$  N,  $136.1^\circ$  W), top right ( $12.0^\circ$  N,  $121.0^\circ$  W), bottom left ( $16.3^\circ$  S,  $119.9^\circ$  W), bottom right ( $5.6^\circ$  S,  $105.2^\circ$  W).

The bore wave is located in a rather restricted portion of the Tharsis region of Mars, bordered on the north and south by latitudes  $0^\circ$  and  $-10^\circ$  and on the east and west by longitudes  $110^\circ$  and  $120^\circ$ , respectively. The south-west and north-east corners of this square are marked by two of the large martian shield volcanoes, Arsia Mons and Pavonis Mons. The south-east corner touches the western end of the Valles Marineris canyon complex while the small volcano Ulysses Patera stands at the north-west corner. The surface in the region is a level plateau roughly 9 km above the martian reference level. The fact that the bore wave seems to have been seen whenever this area has been monitored around solstice strongly suggests that it is a daily occurrence there.

**Table 1** Bore wave observations

|      | Rev         | Mars time | $L_s$ |
|------|-------------|-----------|-------|
| 1976 | 39A01, 02   | 0700      | 101.1 |
|      | 40A30, 35   | 0630      | 101.5 |
|      | 40A86–95    | 0645      | 101.5 |
|      | 41A55       | 0830      | 102.0 |
|      | 62A14–17    | 0630      | 111.8 |
|      | 62A21–25    | 0700      | 111.8 |
|      | 62A31–34    | 0730      | 111.8 |
| 1978 | 701A50      | 0718      | 88.5  |
|      | 735A13      | 0649      | 103.6 |
|      | 735A18–33   | 0820      | 103.6 |
|      | 759A87, 88  | 0730      | 114.8 |
|      | 774A11      | 0630      | 121.5 |
|      |             |           |       |
| 1980 | 334S 10, 12 | 0830      | 69.8  |
|      | 334S 20, 22 | 0833      | 69.8  |
|      | 402S01–04   | 0615      | 99.6  |
|      | 05–08       | 0830      | 99.7  |
|      | 09–12       | 1015      | 99.7  |
|      | 404S05–08   | 0545      | 100.5 |
|      | 09–12       | 0630      | 100.5 |
|      | 21–34       | 0715      | 100.6 |
|      | 62–65       | 0945      | 100.6 |
|      | 408S31–34   | 0630      | 102.4 |
|      | 41–44       | 0845      | 102.4 |
|      | 51–54       | 1030      | 102.5 |
|      | 412S43–44   | 0645      | 104.2 |
|      | 51–54       | 0845      | 104.3 |
|      | 61–64       | 1045      | 104.3 |
|      |             |           |       |

The times given are an average for the set of images referenced. The areocentric longitude,  $L_s$ , is measured from  $0^\circ$  to  $360^\circ$  throughout the martian year, so that  $90^\circ$  is the length of a season.  $0^\circ$  represents northern spring equinox.

An example of the bore wave is shown in Fig. 1; the sharp, gently curved boundary is roughly perpendicular to the axis of the Tharsis Ridge. The front spans the diagonal of the square described above and is  $\sim 1,000$  km long. Note the branch to the east, which is joined to the main portion by a link at the Claritas Fossae Ridge. On other orbits, bifurcations are observed in the bore wave at various locations.

The bore wave phenomenon appears most clearly as a well defined discontinuity, or front, in the earliest morning views; occasionally, as on rev. 40A (ref. 1, Fig. 9) two distinct fronts are visible, the southern one having a greater curvature than the northern. As the morning progresses, the bore wave generally becomes a system of parallel, evenly spaced waves resembling a wave train, particularly to the west of the volcanoes, such as that shown in Fig. 2. The wavelength is typically 10–20 km, and there are generally about 10 waves in the wave train. As the waves encounter Pavonis Mons, they sometimes form two distinct branches to either side of the peak, as seen in Fig. 2. This clearly indicates that the atmospheric phenomenon is confined to the



layers near the surface of the plateau, well below the volcano's summit. Cloud remnants have been detected as late as noon, although these generally disappear by 10.00–10.30 local time (LT). (The martian day or sol is 24 h 39 min 35 s long. The equivalent system used here (to which local time refers) is to divide the sol into 24 'h' and 60 'min'. Universal time refers to terrestrial hours and minutes and velocities are determined in this system.)

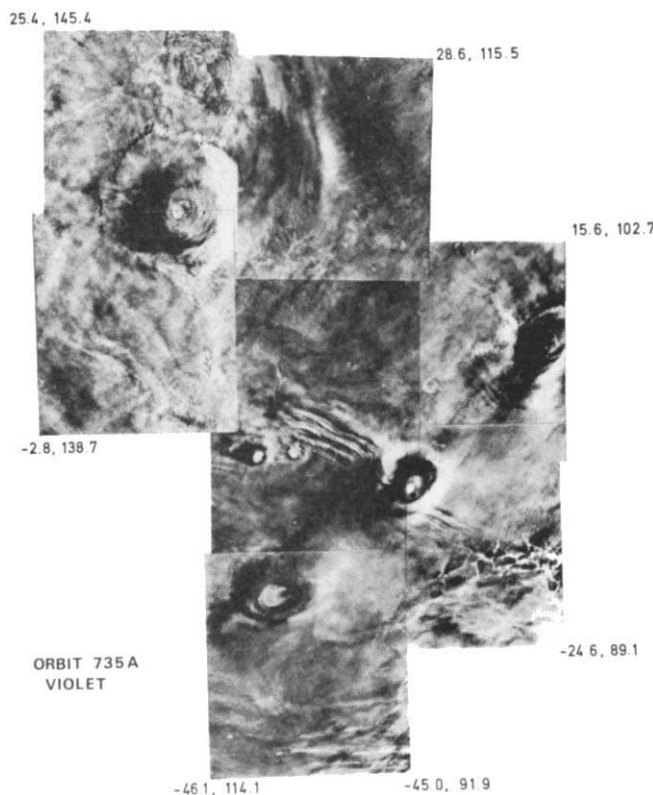
The seasonal dependence is similar to that of the general cloud activity in the region<sup>2</sup>. In particular, the saturation of the morning surface layers with water vapour during this season permits condensation which marks meteorological phenomena, such as waves, that might otherwise go unnoticed. Seasonal changes also occur in the wind direction, inasmuch as the predominant wind streaks vary with season, which may be related to the changes in atmospheric conditions.

A similar, but much less dramatic, phenomenon occurs in the north-east between Pavonis Mons and Ascræus Mons, but evidence is more fragmentary; there is also some evidence for a similar feature in Elysium. Thus, the bore wave may not be unique to the Tharsis region, but is most prominent there.

Table 1 lists all known bore wave observations. Table 2 shows velocities determined for days on which sets of pictures were obtained; the determined speeds depend on location along the wavefront and on time. The average speed was  $21.0 \text{ m s}^{-1}$  over five orbits during the time intervals shown in the western section of the bore wave near Ulysses Patera. This speed slowed to  $6.8 \text{ m s}^{-1}$  for one observation at 09.56 on orbit 404S.

In the central region, between the two large shield volcanoes, the average speed was  $13.1 \text{ m s}^{-1}$  while that in the eastern branch was  $19.1 \text{ m s}^{-1}$ . Velocities were determined by measuring from the leading clouds in the wave train. However, complications arose as to the position of the corresponding clouds especially in the third set of images, as it is possible that the leading clouds had already dissipated, leaving a visible leading edge which corresponds to an intermediate position of the wave train. This is suggested in Fig. 3, where, by the third sequence, only part of the western wave train is visible, and the calculated speed was considerably reduced.

Figure 3 shows the motions of the bore wave complex monitored on rev. 404S. Each observation is indicated by a shading scheme and an associated time, with the prominent clouds illustrated by a heavy line. Area A is relatively unobstructed except for the low profiles of the minor volcanoes. The topography of region B is represented by a trough 16 km deep between the southernmost volcanoes of the Tharsis Ridge. Roth



**Fig. 2** Olympus Mons and the Tharsis Ridge. Mosaic composed of pictures obtained on rev. 735 of Viking Orbiter 1. The season is almost identical with that of Figs 1 and 3,  $L_s = 103.6^\circ$ , but these views were obtained one martian year earlier. Local time 08.20. The view illustrates the 'breaking' of the tidal bore when it encounters Pavonis Mons.

*et al.*<sup>3</sup> suggest that area C has a more complex relief than is suggested by the currently available topographical maps. A narrow, deep passage exists between Noctis Labyrinthus at 10 km and Claritas Fossae at 9 km above the mean martian radius, which traps the wave form.

The average speed in region A of Fig. 3 of the cloud associated with the bore wave is  $19.5 \text{ m s}^{-1}$ . However, the average between the second and third sequence has slowed to  $6.8 \text{ m s}^{-1}$ . Times in Fig. 3 are approximately at the longitude shown, whereas times shown in Tables 1 and 2 are calculated at different longitudes. In area B, the motion is complicated by upward motion on the southern flanks of Pavonis Mons and waves seen in Fig. 1 occur ahead of the principal bore wave cloud. As seen in Table 2, bore wave motions are considerably smaller than those further west. Further east, near Noctis Labyrinthus, smaller speeds are detected. However, the local time across the bore wave from west to east is  $> 1 \text{ h}$ , and the reduced speeds in the eastern branch are consistent with the hypothesis that the bore wave decelerates. In region C, the wave is bent as well as branched.

The season when the bore wave has been observed is characterized by extensive and diverse clouds in the Tharsis region<sup>2</sup>. The early morning hours are characterized by extensive radiation fogs and by ubiquitous, and sometimes spectacular, instability waves similar to terrestrial billows. These morning condensations are undoubtedly associated with the low thermal inertias in the region<sup>4</sup> which cause enhanced nocturnal cooling in the layers of the atmosphere adjacent to the plateau. In the afternoon, convection, enhanced by thermal forcing due to the same low inertia, causes the formation of extensive cloud cover to the west of the volcanic peaks.

The low thermal inertia of Arsia Mons and its relatively large horizontal extent is probably responsible for the bore wave

**Table 2** Bore wave motions

| Rev                            | Mars time |       |       | Distance moved (km)   |     | Average velocity<br>(m s <sup>-1</sup> ) |      |
|--------------------------------|-----------|-------|-------|-----------------------|-----|------------------------------------------|------|
|                                | 1         | 2     | 3     | 1-2                   | 2-3 | 1-2                                      | 2-3  |
| Ulysses Patera                 |           |       |       |                       |     |                                          |      |
| 735A                           | 06.49     | 08.20 |       | 150                   |     | 23.9                                     |      |
| 402S                           | 06.00     | 08.09 |       | 143                   |     | 17.9                                     |      |
| 404S                           | 06.33     | 07.05 | 09.56 | 54                    | 75  | 19.5                                     | 6.8  |
| 408S                           | 06.21     | 08.20 |       | 210                   |     | 27.6                                     |      |
| 412S                           | 06.47     | 08.28 |       | 117                   |     | 16.3                                     |      |
| Average velocity (1-2)         |           |       |       | 21.0 (5 measurements) |     |                                          |      |
| Area between Pavonis and Arsia |           |       |       |                       |     |                                          |      |
| 402S                           | 06.31     | 08.47 |       | 102                   |     | 12.8                                     |      |
| 404S                           | 06.33     | 07.39 |       | 37                    |     | 13.3                                     |      |
| Average velocity (1-2)         |           |       |       | 13.1 (2 measurements) |     |                                          |      |
| Western Noctis Labyrinthus     |           |       |       |                       |     |                                          |      |
| 404S                           |           | 07.39 | 10.46 |                       | 111 |                                          | 10.1 |
| 412S                           | 06.47     | 08.28 |       | 137                   |     | 19.1                                     |      |
| Average velocity (1-2)         |           |       |       | 19.1 (1 measurement)  |     |                                          |      |

Motion of bore wave in three distinct regions with LT averaged for the set of images referenced. A correction is applied to these times between events for longitude changes in calculating velocities.

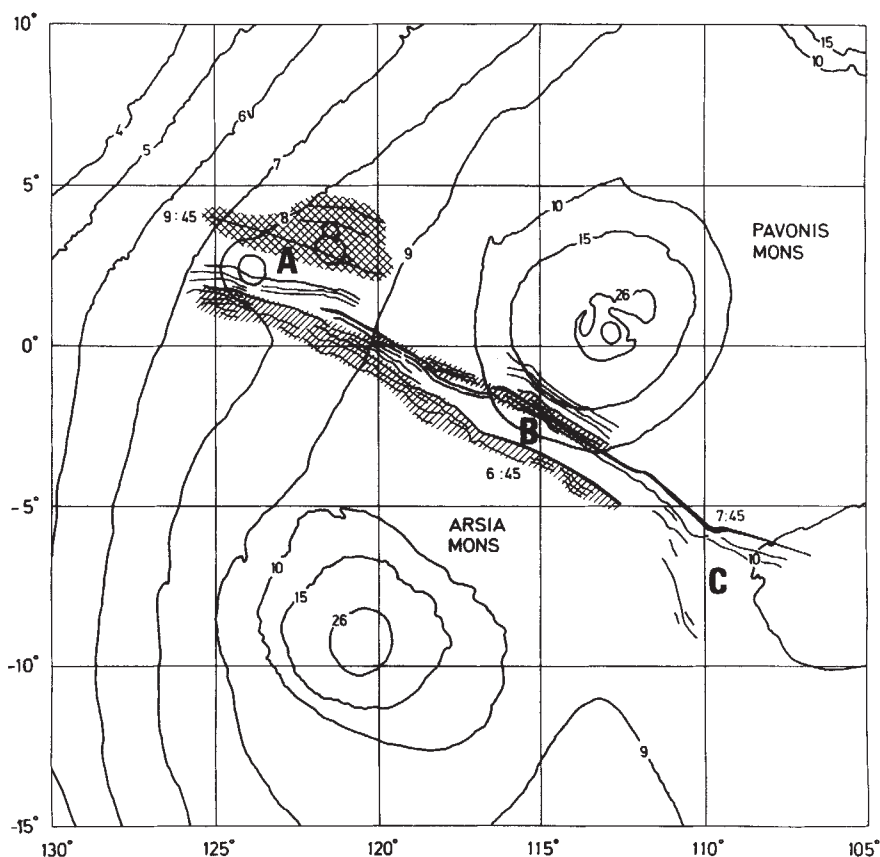
phenomenon. We propose that the feature is a front which separates a layer of air descending from Arsia Mons from the air native to the region. The descending currents are produced by the extreme cooling of air layers adjacent to the Arsia shield which has the lowest thermal inertias observed on the planet. The same downslope winds have been associated with the dust clouds commonly seen to the south-east in Solis Planum in a different season<sup>5</sup>. The region of minimum thermal inertia is centred on Arsia Mons; pre-dawn temperatures there are roughly 138 K during this season, which is 34 K below the mean global temperature at this local time. The atmosphere in the 20–30 km region, corresponding to the Arsia summit, shows little diurnal variation; temperatures of  $\sim 170$  K are expected (based on IRTM  $15\text{ }\mu\text{m}$  measurements). Figure 4 displays 20, 15, 11, 9, 7  $\mu\text{m}$  temperature contours (K) corresponding to the LT of Fig. 2. Although the structure of the atmosphere below 20 km was not precisely measured, it is likely that a thermal inversion occurs near the surface of the plateau due to the inertial effects described above. Between the top of this inversion layer and 20 km, the morning lapse rate is subadiabatic, as seen from the Lander 2 entry profile<sup>6</sup>. Temperatures measured by the other IRTM channels, also shown in Fig. 4, are essentially ground temperatures. The fact that  $T_9$  is larger than  $T_7$  and  $T_{11}$  indicates that the clouds in Fig. 2 are condensate<sup>7</sup>.

The surface layers of the atmosphere cool during the night and the resulting downward motion produces a katabatic wind. Mountain and valley breezes generated by diurnal thermal effects are a terrestrial analogue; similar effects are responsible for the sea and land breezes in coastal regions. The effects are magnified on Mars by the unusually large thermal contrasts in the Tharsis region. The front which marks the discontinuity in air masses is similar to the terrestrial frontal features which accompany the sea breezes.

Wave trains occur along the front after dawn when the 'tide' of mountain air should be starting to diminish. It is possible, therefore, that the waves are internal gravity wave solitons, as suggested by Leovy<sup>8</sup>. As mentioned above, the atmospheric state at this time is quite conducive to gravity wave effects. However, to excite such a wave, the large extent of the wave front would require a ridge across the entire Tharsis plateau perpendicular to its axis which is not observed in images of the region.

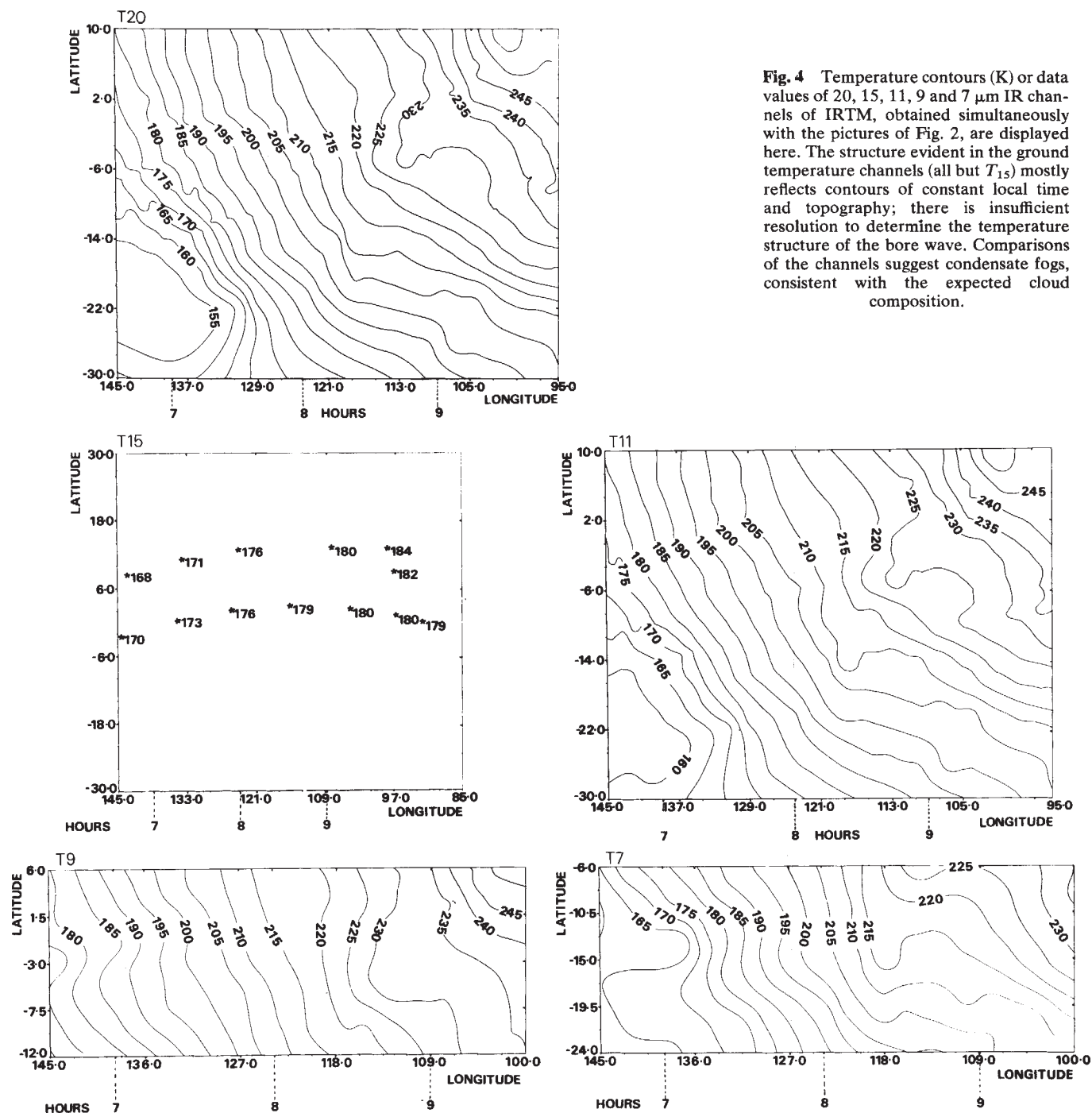
Alternatively, oscillations could be produced by the vertical component of parcel velocity acquired during descent; or the waves could be some sort of a compressional feature formed in front of the rapidly moving air mass. The hypothesis for the bore wave presented here is supported by the visual evidence. Global views of the front appear to centre it on Arsia Mons, except for the section to the east of Claritas Fossae. Deviations from circularity are probably caused by the non-symmetric topography of Arsia<sup>7</sup>. Similar downslope currents from Pavonis should, because of the volcano's smaller size, be less pronounced but they could tend to flatten the front and slow its advance in the region between the volcanoes as observed. Interactions with global circulation and thermal tides, variations in roughness and small-scale topography would produce asymmetries.

The descending currents are considerably drier than the air prevalent on the Tharsis Ridge. The latter is saturated at night, and extensive fogs are observed. It would be expected that fogs would not readily form behind the front because of the relative dryness of the air. Some of the pictures do suggest such a contrast; because the layer of drier air may not reach the surface, fogs could still exist near the surface making generalizations difficult. By noon LT, the surface is heated sufficiently to produce convection in the lowest layers and during this season extensive clouds with a cellular structure may form near the volcanoes. This is evident on orbit 404S (404S67–68). Briggs *et*



**Fig. 3** Motions of the bore wave complex, monitored at universal time difference of about 45 min and 3 h on rev. 404S of Viking Orbiter 1. The primary fronts are shown as heavy lines. The USGS topographical map of the Tharsis Ridge with contours in km is used for reference with north at the top. Region A is a relatively unobstructed region with a few minor volcanoes. Region B is represented by a wide trough 16 km deep, while region C is a region of complex topography. The local times of the individual bore waves are indicated on the diagram together with an indication of their flow in a generally northerly direction.





**Fig. 4** Temperature contours (K) or data values of 20, 15, 11, 9 and 7  $\mu\text{m}$  IR channels of IRTM, obtained simultaneously with the pictures of Fig. 2, are displayed here. The structure evident in the ground temperature channels (all but  $T_{15}$ ) mostly reflects contours of constant local time and topography; there is insufficient resolution to determine the temperature structure of the bore wave. Comparisons of the channels suggest condensate fogs, consistent with the expected cloud composition.

*al.*<sup>1</sup> suggest that during the afternoon unslope winds lead to adiabatic cooling of the air and condensation of the water vapour, forming a phenomenon known as the W or M cloud.

The position of the bore wave would seem to be due to the large volume of Arsia relative to Pavonis and Ascraeus as well as to the somewhat lower thermal inertia of the Arsia region. However, downslope currents should exist on all mountains and as mentioned above, there is some evidence for similar, though much weaker, features associated with the other shield volcanoes on Mars. Interactions between the winds from Arsia and Pavonis currents may explain the shape and some structure, such as branching, in the front.

Thus, the bore wave is qualitatively consistent with a thermally induced diurnal katabatic breeze. The branch to the east of Claritas Fossae, to the south of Noctis Labyrinthus, in the vicinity of 15°S 105°W, is the hardest to understand. As the front terminates at the ridge, the eastern branch may be locally enhanced from the Noctis Labyrinthus region, which is 2 km

higher than the Solis Planum region to the south with the topographical contours running NNW-SSE, roughly parallel to the eastern branch. It is also possible that downslope winds from both of the southerly Tharsis volcanoes combine to produce the Syria Planum branch.

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# Surface temperature sensitivity to increased atmospheric CO<sub>2</sub>

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The atmospheric CO<sub>2</sub> content has increased significantly over recent decades, mostly as a result of the burning of coal and oil; and it could double in less than a century<sup>1-4</sup>. Because the CO<sub>2</sub> molecule absorbs radiation in the thermal long-wavelength domain, it contributes to the blanketing or greenhouse effect of the atmosphere, and an increase in atmospheric CO<sub>2</sub> would be expected to lead to a rise in the mean surface temperature of the Earth<sup>1-6</sup>. Early estimates indicate that for doubled CO<sub>2</sub>, the surface temperature would be raised by 1–10 K, but recent numerical experiments with radiative-convective or general circulation models (RCM, GCM) of the atmosphere seemed to narrow this range to 2–4 K (see refs 2–6). However, Newell and Dopplack<sup>7</sup>, and Idso<sup>8</sup>, argue that these results are inconsistent with the observed or computed values of certain terms of the surface energy budget, which seem to indicate a far smaller sensitivity. Here, I examine all of the terms of the surface energy budget<sup>7-11</sup> and find that there is no basic disagreement between the surface energy budget approach and the more elaborate model experiments, provided that there is a clear distinction between external perturbation and atmospheric feedback, and that equivalent representations of surface processes are used. However, the temperature and humidity dependence of the terms of the surface energy budget shows that the surface temperature sensitivity depends critically on the way in which the atmospheric humidity evolves, and on the degree of compensation in the perturbation of the latent and sensible heat fluxes.

If the Earth's atmosphere were in radiative equilibrium, it would be relatively straightforward to evaluate the differential greenhouse effect of the added CO<sub>2</sub>. However, the steepening of the atmospheric temperature gradient is limited by the possibility of nonradiative transport of sensible and especially latent heat. As far as the surface is concerned, the greenhouse is 'drafty', and so the CO<sub>2</sub> heating is distributed throughout the atmosphere.

In terms of the absorbed solar flux  $A$ , net upward longwave flux  $F$ , and net upward sensible and latent heat fluxes  $S$  and  $LE$  (where  $L$  is the latent heat of vaporization in J kg<sup>-1</sup> H<sub>2</sub>O and  $E$  is the rate of evaporation in kg H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>), the surface energy budget is:

$$A = F + S + LE \quad (1)$$

Doubling the CO<sub>2</sub> increases the long-wavelength optical thickness of the atmosphere and its downward long-wavelength emission, in the absence of any other changes. This is the proper external perturbation of the system,  $X = 1.3 \text{ W m}^{-2}$ , which must be distinguished from radiation feedback terms,  $\Delta F$ , due to changes in atmospheric temperature and humidity; these can be substantially larger<sup>12</sup>. Estimates by Ramanathan<sup>4</sup> and by Newell and Dopplack suggest  $X = 1.3 \text{ W m}^{-2}$ . The perturbation equation is:

$$X = -\Delta A + \Delta F + \Delta S + \Delta LE \quad (2)$$

The reciprocal surface temperature sensitivity  $\lambda \equiv X/\Delta T$  is then given by:

$$\lambda = \lambda_0 + \lambda_1 \eta \quad (3)$$

$$(\lambda_0, \lambda_1) = (\partial/\partial T, \partial/\partial H)(-A + F + S + LE) \quad (4)$$

and  $\eta = \Delta H/\Delta T$  describes how the absolute humidity (vapour pressure) near the surface changes with temperature. I assume here that  $\Delta A = 0$ : although both cloud and ice-albedo feedbacks may be major factors in the ultimate climate response, they are irrelevant to the present discussion.

Newell and Dopplack<sup>7</sup> and Idso<sup>8</sup> refer to the surface energy budget and give results which imply a low surface temperature sensitivity (high  $\lambda$ ), Idso relies on experimental data, Newell and Dopplack note that the term  $\partial LE/\partial T$  dominates  $\lambda_0$  and must be of the order of  $10 \text{ W m}^{-2} \text{ K}^{-1}$  or higher. In RCM experiments, one-dimensional models of the global mean atmospheric structure with height are constructed for different values of external parameters such as the solar constant, atmospheric CO<sub>2</sub> content, or surface albedo; moreover, the absolute or relative humidity distribution is also treated as an independently variable parameter of the system. With the convective adjustment procedure, radiative equilibrium is imposed ( $LE = S = 0$ ) in those layers in which the lapse rate is subcritical; elsewhere the sum ( $LE + S$ ) is implicitly determined as satisfying zero total energy flux divergence, the total radiative flux divergence being determined by the assumed critical lapse rate. From the results of RCM experiments carried out at fixed absolute humidity ( $\Delta H = 0$ ), the value of  $\partial(LE + S)/\partial T$  at the surface can be extracted. In GCM experiments, three-dimensional time-dependent models of the atmosphere are used, and values of different atmospheric quantities are computed for different sets of external parameters by taking means over sufficiently long runs of simulated weather. The global mean surface values of  $S$  and  $LE$  are available, separately, but the contributions  $\partial/\partial T$  and  $\partial/\partial H$  to  $\lambda$  cannot be separated, because  $H$  is not an independent parameter of the calculation.

The contribution of the longwave flux feedback  $\Delta F$  to  $\lambda_0$  and  $\lambda_1$  can be evaluated using a version of Brunt's formula<sup>13</sup>:

$$F = \sigma T^4 - F\downarrow = \sigma T^4(a - b\sqrt{H}) \quad (5)$$

This implies that the relative distributions of temperature and humidity with height are not substantially changed by the external CO<sub>2</sub> perturbation, and that they are similar to those from which Brunt's empirical formula was derived. This is consistent with the troposphere as a whole being heated. Cooling of the stratosphere has little direct effect on the downward longwave flux  $F\downarrow$  at the surface, although it is an essential part of the maintenance of global energy balance. Adopting  $a = 0.36$  and  $b = 0.06$  yields values of  $F$ ,  $\partial F/\partial T$  and  $\partial F/\partial H$  consistent with other estimates<sup>7,13-16</sup> (Tables 1 and 3). For fixed relative humidity  $h = 0.77$  and temperature 288 K,  $dF/dT = -2.0 \text{ W m}^{-2} \text{ K}^{-1}$ ; without feedback in other terms of the surface energy budget, this would lead to  $\lambda < 0$  and an unstable situation<sup>14</sup>.

Idso<sup>8</sup> has suggested that 'natural experiments' can be used to estimate surface temperature response, dividing an observed change in surface temperature by an observed change in downward long-wavelength flux. Such a procedure yields a quantity  $\lambda' = (X + \Delta F\downarrow)/\Delta T$  or  $\lambda' = \lambda + \partial F\downarrow/\partial T \approx \lambda + 4.6 \text{ W m}^{-2} \text{ K}^{-1}$  using equation (5), so that for  $\lambda = 0.5$ ,  $\lambda' \approx 5$ . Failure to distinguish between external perturbation  $X$  and atmospheric feedback  $\Delta F\downarrow$  leads to an estimate of surface temperature

**Table 1** Observed and computed values of the terms of the surface energy budget

| $X$  | $A$                    | $F$  | $LE$ | $S$  | Observed                    |      |     |
|------|------------------------|------|------|------|-----------------------------|------|-----|
| 0    | 157                    | 52   | 88   | 17   | Ref. 16                     |      |     |
| 0    | 166.0                  | 63.5 | 75.4 | 27.2 | Standard, ref. 19           |      |     |
| 1.3  | 165.3                  | 60.6 | 80.9 | 25.1 | 2×CO <sub>2</sub> , ref. 19 |      |     |
| Case | Computed in this paper |      |      |      | $T$                         | $h$  | $C$ |
| 1    | 154.4                  | 55.8 | 78.4 | 20.2 | 288                         | 0.77 | 20  |
| 2    | 133.7                  | 47.3 | 57.8 | 28.6 | 291                         | 0.80 | 14  |
| 3    | 180                    | 18.2 | 71.3 | 4.0  | 300                         | 0.80 | 10  |

Units:  $A, F, LE, S$  (W m<sup>-2</sup>);  $T$  (K);  $C$  (W m<sup>-2</sup> mbar<sup>-1</sup>);  $h = H/H_s$ .

Case 1, standard global mean conditions; case 2, gives greater weight to warmer oceans with lower wind speeds.  $F, LE$  and  $S$  are computed using equations (5), (6) and (7) respectively, with  $C$  as shown, while  $A$  is obtained from the surface energy balance condition (equation (1)). Case 3 is given as a representation of a tropical sea surface (the Newell and Dopplack case);  $F$  and  $LE$  are computed as above, but  $A$  and  $S$  are set arbitrarily; note that energy balance is not satisfied.



**Table 2** Temperature sensitivity, for fixed absolute humidity, of the terms of the surface energy budget

| Case | $-\partial A/\partial T$ | $\partial F/\partial T$ | $\partial LE/\partial T$ | $\partial S/\partial T$ | $\gamma'$ | $\lambda_0$ |
|------|--------------------------|-------------------------|--------------------------|-------------------------|-----------|-------------|
| 1    | 0                        | +0.77                   | +22.0                    | -8.4                    | 0.38      | 14.4        |
| 2    | 0                        | 0.65                    | 18.2                     | -6.9                    | 0.38      | 12.0        |
| 3    | 0                        | 0.24                    | 21.0                     | 0                       | 0         | 21.2        |
| 4    | 0                        | 1.38                    | 33.3                     | +2.0                    | 0         | 36.7        |
| 5    | 0                        | 0.8                     | 20                       | -20                     | 1         | 0.8         |
| 6    | 0                        | -11                     | 20                       | -8                      | 0.4       | 1           |

(Units:  $\text{W m}^{-2} \text{K}^{-1}$ .)

Cases 1, 2, 3, as in Table 1, assuming  $\partial S/\partial T = 0$  for case 3. Cases 4 and 5 represent my interpretation of the Newell and Dopplack<sup>7</sup> and Manabe and Wetherald<sup>15</sup> results respectively, while case 6 represents an extreme case of what might be called condensation-radiation feedback.

sensitivity that is an order of magnitude too small. The results of Idso's natural experiments do not contradict model predictions.

The dominant contributions to  $\lambda_0$  and  $\lambda_1$  come from terms  $LE$  and  $S$ . The usual parametrization for  $LE$  is:

$$LE = C(H_s - H) \quad (6)$$

where coefficient  $C$  is proportional to wind speed, and  $H_s$  and  $H$  are the saturation and atmospheric vapour pressures respectively<sup>7</sup>. The sensible heat flux  $S$  can similarly be taken to be proportional to the temperature gradient near the surface; the steepness of this gradient is limited by the latent heat flux<sup>17,18</sup>. This behaviour can be represented approximately by  $S = S_0 - \gamma' LE$ , or

$$S + LE = S_0 + C(1 - \gamma')(H_s - H) \quad (7)$$

In their  $\text{CO}_2$  doubling GCM experiments, Manabe and Wetherald<sup>19</sup> found  $\Delta S/\Delta LE = -0.38$ . With this value for  $\gamma'$ , the surface energy budget terms (compared in Table 1 with semi-empirical estimates<sup>6</sup>) are consistent with  $C$  of the order of  $10\text{--}20 \text{ W m}^{-2} \text{ mbar}^{-1}$ , as are the contributions of these terms to  $\lambda_0$  and  $\lambda_1$  (Table 3). These are an order of magnitude larger than the radiation feedback terms.

For fixed absolute humidity,  $\lambda = \lambda_0 \approx 10\text{--}15 \text{ W m}^{-2} \text{ K}^{-1}$ . This is roughly consistent with the Newell and Dopplack estimate of  $\lambda_0 \approx 30 \text{ W m}^{-2} \text{ K}^{-1}$  over tropical seas where latent heat liberation dominates. Their value of  $20 \text{ W m}^{-2} \text{ K}^{-1}$  takes into account the fraction of tropical latitudes covered by desert regions where sensible heat flux dominates and  $\lambda_0 \approx 2 \text{ W m}^{-2} \text{ K}^{-1}$ . As they assume  $S$  and  $LE$  are independent and not competing fluxes, they implicitly assume  $\gamma' = 0$ . In our formulation, partial compensation in latent and sensible heat flux sensitivity to temperature does slightly reduce  $\lambda_0$ , thus enhancing the surface temperature sensitivity. These values remain in discord, however, with the value  $\lambda_0 \approx 1 \text{ W m}^{-2} \text{ K}^{-1}$  found by Manabe and Wetherald<sup>15</sup> using an RCM with fixed absolute humidity. Considering equation (2) and noting  $\Delta A = 0$ ,  $\partial(LE + S)/\partial T = \lambda_0 - \partial F/\partial T \approx -0.2 \text{ W m}^{-2} \text{ K}^{-1} \approx 0$  if  $\lambda_0 = 1$ . There is nearly complete cancellation of changes in  $LE$  and  $S$ , that is  $\gamma' \approx 1$ , in a sense the opposite of what Newell and Dopplack assumed. This implicit parameterization of the nonradiative transport terms is probably unrealistic and inherent in the conditions of fixed absolute humidity and convective adjustment to a fixed critical lapse rate (such as  $6.5 \text{ K km}^{-1}$ ). Because these conditions fix both the distribution of temperature and that of longwave absorber/emitters in the lower atmosphere, they leave no room for significant changes in the net upward longwave flux, and because the sum of the radiative and nonradiative fluxes must be constant, with  $\Delta A = 0$ , then  $\Delta(LE + S)$  must also be 0. Various estimates of terms contributing to  $\lambda_0$ , illustrating the role of  $\gamma'$ , are shown in Table 2.

In the above argument, no explicit distinction is made between the surface temperature  $T_s$  and the near-surface air temperature  $T_a$ . Ramanathan<sup>4,24</sup> has noted that the downward longwave flux is substantially enhanced as a result of the increase in air temperature due to condensation of the additional water evaporated as surface temperature rises. This effect is

incorporated in our evaluation of  $\partial F/\partial T$ , which assumes  $T_s = T_a = T$ . A strongly negative  $\Delta F$  would help to cancel the strongly positive  $\Delta LE$ , as shown in Table 2. This would require that  $T_s - T_a$  should fall as both temperatures rise, which would be the case if most of the additional condensation occurred fairly low in the atmosphere; in such conditions equation (5) underestimates the magnitude of  $\Delta F$ . However, I doubt that this can explain the low value for  $\lambda_0$  given by Manabe and Wetherald<sup>15</sup>, because it requires  $\Delta F \approx -11 \text{ W m}^{-2}$ , with  $\Delta H = 0$ , while even with  $\Delta H = +3 \text{ mbar}$  and a GCM representation of atmospheric heating by condensation, the later result<sup>19</sup> was  $\Delta F = -3.1 \text{ W m}^{-2}$ .

Fixed absolute humidity ( $\eta = 0$ ) is extremely unlikely, as it requires very high sensitivity of the precipitation rate  $P$ : either  $\partial P/\partial T$  or  $\partial P/\partial H$ , or both, must be strongly positive. However, the assumption of fixed relative humidity ( $\eta = \eta_r$ ), although reasonable<sup>14</sup>, remains arbitrary<sup>20</sup>. Note that for some cases, fixed relative humidity cannot be maintained:  $\lambda = 0$  for a critical value  $\eta = \eta_c < \eta_r$ . The Manabe and Wetherald<sup>15</sup> results may have suggested that the difference between the two cases ( $\eta = 0$ ,  $\eta = \eta_r$ ) was modest, but the above analysis shows that this depended on the unrealistic implicit assumption  $\gamma' = 1$ . Therefore, the exact value of  $\eta$  is needed, as the reciprocal temperature sensitivity  $\lambda = \lambda_0 + \lambda_1 \eta$ , and  $\lambda_0$  and  $\lambda_1$  are opposite in sign and of comparable (large) magnitude.

In the Manabe and Wetherald GCM experiments<sup>19</sup>, relative humidity near the surface rises by a few per cent when the  $\text{CO}_2$  doubles or when the solar constant increases by a few per cent. Using the NCAR GCM, Schneider *et al.*<sup>21</sup> found much smaller increases (0.57%) in relative humidity near the surface, accompanying a rise of 2 K in surface temperature; relative humidity in higher layers, together with cloud area, fell by a few per cent, even though evaporation, precipitation, absolute humidity and liquid water content all increased. Roads<sup>22</sup> noted the influence of the model large-scale saturation limit: when it is set at 100%, higher relative humidities are reached than when it is reduced to 85%. Clearly a GCM will tend to maintain fixed relative humidity when this is close to the saturation limit adopted originally.

Results of GCM experiments, indicating a reciprocal surface temperature sensitivity  $\lambda = 0.5 \text{ W m}^{-2} \text{ K}^{-1}$ , can be interpreted as revealing nearly complete cancellation of much larger terms related to the nonradiative energy fluxes at the surface. It would be easier to understand the GCM results, if the experimenters reported (as did Manabe and Wetherald<sup>19</sup>) global mean values of the components of the surface energy balance, and gave more details on the parameterizations used for such critical processes as evaporation, sensible heat transport and condensation. We

**Table 3** Combined temperature and humidity sensitivity of the terms of the surface energy budget

| $f =$                                | $F$   | $LE$  | $S$   | $\lambda_0, \lambda_1; \lambda$ | $\Delta T$ |
|--------------------------------------|-------|-------|-------|---------------------------------|------------|
| <b>Case 1</b> $\partial/\partial T$  | +0.77 | +22   | -8.4  | +14.4                           |            |
| $\partial/\partial H$                | -3.23 | -20   | +7.6  | -15.6                           |            |
| $\phi_f(\eta = 0.80) =$              | -1.81 | +6.0  | -2.32 | +1.9                            | 0.7        |
| $\phi_f(\eta_r = 0.85) =$            | -1.98 | +5.00 | -1.94 | +1.1                            | 1.2        |
| $\phi_f(\eta = 0.91) =$              | -2.17 | +3.80 | -1.48 | +0.15                           | 8.7        |
| <b>Case 2:</b> $\partial/\partial T$ | +0.65 | +18.2 | -6.9  | +12.0                           |            |
| $\partial/\partial H$                | -3.00 | -14.0 | +5.3  | -11.7                           |            |
| $\phi_f(\eta = 0.8) =$               | -1.75 | +7.00 | -2.66 | +2.59                           | 0.5        |
| $\phi_f(\eta = 0.9) =$               | -2.05 | +5.60 | -2.13 | +1.42                           | 0.9        |
| $\phi_f(\eta_r = 1.0) =$             | -2.35 | +4.20 | -1.60 | +0.25                           | 5.2        |
| <b>Case 3</b> $\partial/\partial T$  | +0.24 | +21.0 | 0     | +21.2                           |            |
| $\partial/\partial H$                | -2.58 | -10.0 | 0     | -12.5                           |            |
| $\phi_f(\eta = 1.0) =$               | -2.34 | +11.0 | 0     | +8.68                           | 0.15       |
| $\phi_f(\eta = 1.5) =$               | -3.63 | +6.0  | 0     | +2.37                           | 0.55       |
| $\phi_f(\eta_r = 1.68) =$            | -4.09 | +4.2  | 0     | +0.11                           | 11.8       |

Results given in Fig. 10 of ref. 19 yield

$$\phi_f(\eta = 1.02) \quad -0.99 \quad +1.88 \quad -0.72 \quad +0.41 \quad 2.93$$

with a term  $\phi_{-A} = +0.24$ , corresponding to  $\Delta A = -0.7 \text{ W m}^{-2}$

Units:  $f, F, LE, S$  ( $\text{W m}^{-2}$ );  $T$  (K);  $H$  (mbar);  $\eta$  (mbar  $\text{K}^{-1}$ );  $\lambda, \phi_f(\eta) = \partial f/\partial T + \eta \partial f/\partial H$  ( $\text{W m}^{-2} \text{ K}^{-1}$ );  $\Delta T$  computed for  $X = 1.3 \text{ W m}^{-2}$ .

are still far from realistic parameterizations of these processes, and this casts doubt on the accuracy of the results. Table 3 illustrates how only slight changes in  $\eta$  lead to large differences in  $\lambda$ . The sensitivity may be low. However, the possibility that the temperature sensitivity to  $\text{CO}_2$  increase could be even higher than values currently cited, with a lagged response masked for the time being by random fluctuations<sup>23</sup>, should be investigated.

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At present, depending on the response of the hydrological cycle and of atmospheric humidity to a doubling of  $\text{CO}_2$ , the rise in surface temperature could be as low as 0.5 K, or as high as 10 K.

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## Bicontinuous structure zones in microemulsions

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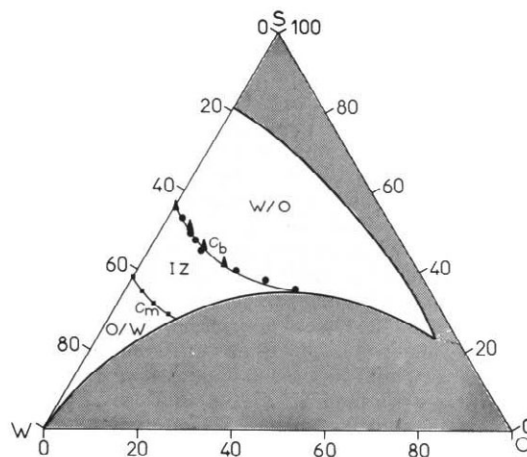
Winsor IV systems<sup>1</sup>, or microemulsions<sup>2</sup>, are macroscopically monophasic fluid transparent compounds made up by mixing water and hydrocarbon in the presence of suitable surface active agents. Depending on composition factors, either water-in-oil (w/o), or oil-in-water (o/w), type systems can be formed. The so-called microemulsions have been intensively investigated<sup>3–8</sup> because of their potential applications as liquid agents in technical processes (for example, enhanced oil recovery, photochemistry and biotechnology). As they belong to the class of short-range organized fluids, microemulsions also concern condensed matter physicochemists interested in elucidating their structural properties<sup>9–12</sup>. That critical phenomena can be observed in microemulsions is confirmed by reports that the electrical conductivity variations with water content increasing in certain w/o microemulsions can be accounted for on the basis of the Percolation and Effective Medium theories<sup>13–16</sup>. It has been suggested<sup>15,16</sup> that percolative conduction reflects disperse aqueous droplet clustering and interlinking processes predictive of a w/o to o/w transition (phase inversion), involving the formation of equilibrium bicontinuous structures described by Scriven<sup>17</sup> as being “related to ordinary liquids as porous media are to homogeneous solids”, and modelled by Talmon and Prager<sup>18</sup> as a random geometry of interspersed water and hydrocarbon domains generated by a Voronoi tessellation. This suggestion is supported by the results reported here, showing that the existence of bicontinuous structures in Winsor IV systems could be identified with the limited composition range separating the w/o and o/w microemulsion regions and over which a post-percolation anomalous conductive behaviour is observed.

Monophasic microemulsions can be defined as populations of minute composite globules with diameters in the range 50–500 Å and suspended in either a hydrocarbon phase in the w/o case or an aqueous phase in the o/w case. The composite globules consist of a mixed surfactant and cosurfactant spherical layer surrounding an internal concentric spherical core made up of either water (w/o), or hydrocarbon (o/w). In a pseudo-ternary phase diagram<sup>7–9</sup>, two main situations can be encountered, depending on composition factors such as the alcohol

conformation<sup>19,20</sup>. For a type of system such as water/SDS/1-pentanol/benzene<sup>19,20</sup> or water/potassium oleate/1-hexanol/hexadecane<sup>21</sup>, the monophasic w/o and o/w areas are disjointed and separated by a region of turbid viscous media. In that case, on increasing the water content, the w/o to o/w transition (phase inversion) occurs through a sequence of structural changes that are detectable on a macroscopic scale and for which the following pattern: w/o globules → w/o cylinders → interspersed water, surface active agents and hydrocarbon lamellar structures → w/o globules has been suggested<sup>21,22</sup>. For a second type of system such as water/potassium oleate/1-butanol/toluene<sup>15,16</sup> or water/SDS/2-methyl-2-butanol/benzene<sup>19,20</sup>, the w/o and o/w areas merge to form a unique transparent monophasic domain that has the shape of a curvilinear triangle, as shown in Fig. 1. In that situation, the w/o to o/w transition is diffuse as no major macroscopic changes are induced, and the phase inversion region cannot be detected and located, as above, either visually or by turbidity tests.

The present experiments show that by studying the electrical conductive behaviour of the second type of microemulsion, it is possible to locate and delineate the w/o to o/w diffuse transition region within which the systems could be in a dynamical equilibrium bicontinuous state.

The microemulsions were made from distilled water and highly pure benzene (Fluka AG, 99%), the cosurfactant being



**Fig. 1** Massic pseudo-ternary phase diagram of the system water/SDS/2Me-2BU/benzene. SDS to 2Me-2BU mass ratio  $k = 1/2$ .  $T = 298$  K. S, 100% combined SDS and 2Me-2BU; W, 100% water; O, 100%  $\text{C}_6\text{H}_6$ . The blank area is the realm of existence of transparent isotropic monophasic systems. For curves  $C_b$  and  $C_m$ , see text.



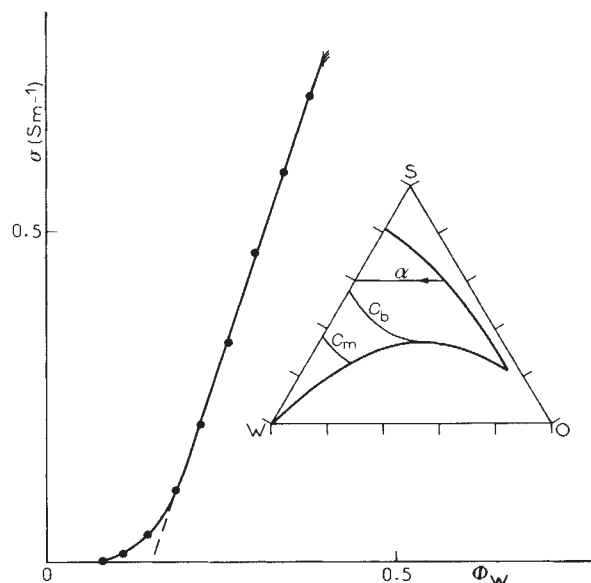


Fig. 2 Variations of  $\sigma$  with  $\phi_w$ . The mass fraction  $p_s$  of combined SDS and 2Me-2BU in the systems is kept at 60%, as illustrated by line  $\alpha$ . General specifications as in Fig. 1.

2-methyl-2-butanol (2Me-2BU), (Fluka AG, 99%) and the surfactant either SDS (Touzard and Matignon, 99%) or potassium oleate (KOL; Fluka AG, technical grade). The surfactant-to-cosurfactant mass ratio  $k$  was kept equal to 1/2 in the case of SDS and 3/5 in the case of KOL. Within the corresponding mass pseudo-ternary phase diagrams, the existence of transparent isotropic monophasic media (blank area in Fig. 1) was determined at room temperature through visual observations of systems whose compositions had been evaluated through accurate weighing. The values of  $\phi_w$ , the volume fraction of water in the systems, were derived from those of the water mass fraction  $p_w$  by means of a digital density meter (Anton Paar DMA 45). Using a Wayne Kerr B 331 autobalance precision bridge and Mullard platinated cells (Philips), determinations of the low-frequency electrical conductivity were carried out at 1.59 kHz and 298 K run by run along composition paths such as line  $\alpha$  in Figs 2 and 3 or line  $\beta$  in Fig. 4. As the system composition varies by addition of water, the mass fraction  $p_s$  of combined surfactant and cosurfactant in the first case ( $\alpha$ -paths), or the mass ratio  $r$  of hydrocarbon to combined surface active agents in the second case ( $\beta$ -paths), remains constant. More details of the experimental procedures are given elsewhere<sup>15,16,19-21</sup>.

Figure 2 shows the variations of the electrical conductivity with  $\phi_w$ , the disperse water volume fraction, recorded on microemulsions in which  $p_s$ , the mass fraction of combined surfactant and cosurfactant, was kept at 60%, as illustrated by line  $\alpha$  on the phase diagram. Similar plots were obtained for values of  $p_s$  ranging from 55% to 80%. The conductive behaviour described by Fig. 2 represents a percolation phenomenon. The characteristic toe at the bottom of the plot can be modelled using a scaling law<sup>23-25</sup>, while the linear upper part of the curve can be depicted through the Effective Medium Theory by means of an equation derived from the general B.B.L.<sup>26-28</sup> expression of the electrical conductivity of binary composites. Percolative conduction phenomena in microemulsions can be explained by assuming the existence of a progressive droplet interlinking and clustering process, as the water content is raised, the conduction mechanism being essentially interfacial<sup>13-16</sup>. As illustrated by Fig. 3, for values of  $p_s$  in the range 0.35–0.55, the conductivity plot recorded along line  $\alpha$  exhibits in its upper part a marked curvature indicating that, past a critical water content, ( $\phi_w = 0.365$  or  $p_w = 0.39$  in the example shown), the variations of  $\sigma$  with  $\phi_w$  do not conform with the Effective Medium Theory model. This phenomenon suggests the existence of a new conduction mechanism reflecting struc-

tural alterations undergone by the microemulsion system at higher water contents. By plotting on the phase diagram of Fig. 1 the points  $M_b$  corresponding to the system compositions computed from the critical values of  $\phi_w$ , it is possible to define the curve labelled  $C_b$  that appears to be an internal boundary of the monophasic region. Figure 4 shows the variations of  $\sigma$  with  $\phi_w$  recorded along line  $\beta$ , that is, on microemulsions in which  $r$ , the mass ratio of benzene to combined surfactant and cosurfactant, was kept at 1/9. Similar plots were obtained for other values of  $r$ . In the example shown, up to values of  $\phi_w = 0.41$  or so (point  $M'_b$ ), the features of the conductivity plot are similar to those of Fig. 2, the toe at the bottom and the medium linear part being characteristic for a percolative conduction in w/o microemulsions. Past this critical water content ( $\phi_w = 0.41$  or  $p_w = 0.435$ ),  $\sigma$  increases more slowly, reaches a maximum (point  $M'_m$ ) for  $\phi_w = 0.60$  ( $p_w = 0.62$ ) and then decreases down to the value of bulk water conductivity that can be considered equal to zero compared with microemulsion conductivity values. It has been demonstrated<sup>19,20</sup> that the descending branch of the  $\sigma$  compared with the  $\phi_w$  curve is typical for o/w microemulsions whose global conductivity decreases because of the progressive dilution of their continuous aqueous phase with water. Consequently, the conductivity curve between points  $M'_b$  and  $M'_m$  indicates the existence of a special conduction mechanism reflecting an intermediary physical state of the system. These results, gained along composition paths such as line  $\beta$  in Fig. 4, are in perfect agreement with those obtained along composition paths such as line  $\alpha$  in Fig. 3, as illustrated by the filled triangles in Fig. 1 which represent system compositions calculated from the critical values of  $\phi_w$  corresponding to the linear/nonlinear transitions (point  $M'_b$ ) observed on conductivity plots such as that of Fig. 4. In addition, points  $M'_m$  (filled squares in Fig. 1), that represent system compositions at which, along  $\beta$ -paths,  $\sigma$  reaches a maximum, define the curve  $C_m$  that seems to be another internal boundary of the monophasic region. Similar conclusions were derived from the study of systems involving as the surfactant KOL instead of SDS, which proves that surfactant nature and purity does not affect fundamentally the phenomena observed.

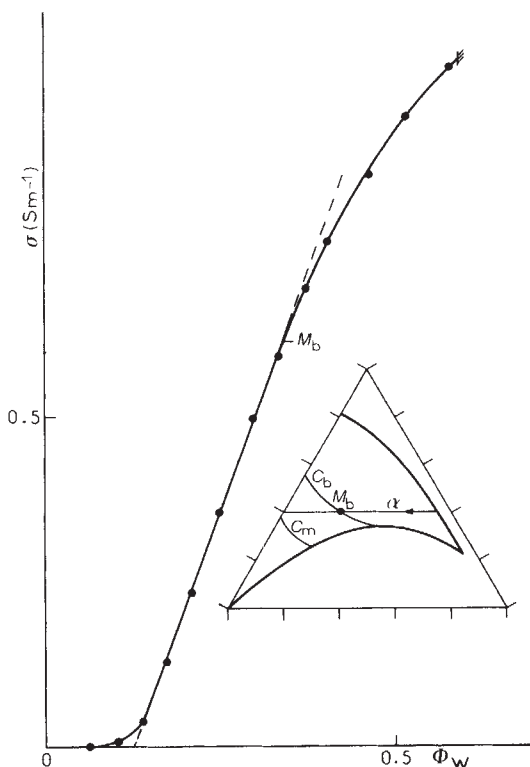


Fig. 3 Variations of  $\sigma$  with  $\phi_w$ . The mass fraction  $p_s$  of combined SDS and 2Me-2BU in the systems is kept at 40%, as illustrated by line  $\alpha$ . General specifications as in Fig. 1.

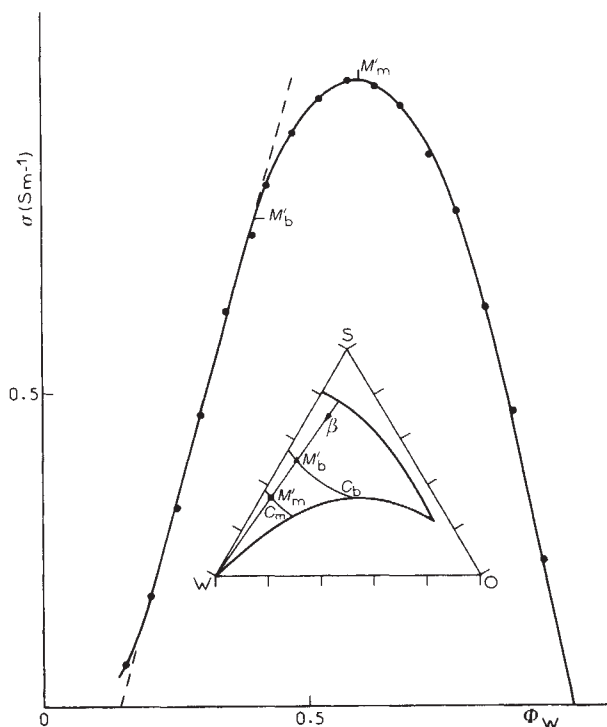


Fig. 4 Variations of  $\sigma$  with  $\phi_w$ . The mass ratio  $r$  of  $C_6H_6$  to combined SDS and 2Me-2BU is kept at 1/9, as illustrated by line  $\beta$ . General specifications as in Fig. 1.

These results show that the unique monophasic region of microemulsion systems of type 2 can be divided into three sub-regions labelled on Fig. 1 W/O, IZ and O/W. The boundaries between the subregions are curves  $C_b$  and  $C_m$  that are defined experimentally as conductivity transition loci. Within W/O, that is, for low water contents, the systems exhibit a percolative electrical conduction (Fig. 2) that can be accounted for by considering droplet interlinking and clustering processes in w/o-type microemulsion<sup>15,16</sup>. Within O/W, that is, for high water contents, the systems are made up of hydrocarbon spheres surrounded by a mixed surfactant and cosurfactant film and suspended in an aqueous phase whose conductivity decreases on addition of water (descending branch of Fig. 4). In the IZ sub-region, bounded by curves  $C_b$  and  $C_m$ , the anomalous conductivity behaviour after the percolation regime (Fig. 3) suggests that the systems are in a hybrid state which is neither w/o nor o/w. IZ can thus be considered as the phase inversion zone over which the w/o to o/w transformation occurs progressively, the systems undergoing no brutal macroscopic alterations, in contrast with the phenomena encountered in the case of type-1 microemulsion systems whose phase diagrams exhibit two disjointed monophasic areas<sup>21,22</sup>. These results strongly support the previous suggestion<sup>15,16</sup> that the model of bicontinuous structures could be applied to the phase inversion phenomenon in Winsor IV systems. It has also been proposed<sup>29</sup>, on the basis of viscosity and conductivity data, that middle-phase microemulsions which are fluid transparent isotropic media in equilibrium simultaneously with an excess water phase and an excess hydrocarbon phase could be assimilated to bicontinuous structures. Should so-called bicontinuous structures be more than a theoretical concept, they deserve thorough investigation as they could represent an unusual type of multi-component organized fluid in dynamical equilibrium. We are hoping to generalize the results reported here by investigating microemulsion conductive behaviour as correlated to phase diagram features and cosurfactant conformation.

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## Palaeomagnetic evidence for a displaced terrain in Western Antarctica

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An outstanding problem in Antarctic tectonics is the relationship between the East Antarctic craton and the late Palaeozoic–Cenozoic orogenic belts that comprise Western Antarctica<sup>1</sup> (Antarctic Peninsula, Ellsworth Land, Eights Coast and Marie Byrd Land; see Fig. 1a). Related problems are the place of Western Antarctica in reconstructions of Gondwanaland and the evolution of the Pacific margin of Gondwanaland. We present here palaeomagnetic data derived from folded Cambrian argillites that crop out in the Ellsworth Mountains which were collected during the 1979–80 austral field season. Although the early Palaeozoic palaeomagnetic field of Gondwanaland is characterized by rapid apparent polar wander, at the present level of coverage, the data are consistent with rotation of the Ellsworth Mountain block with respect to the East Antarctic craton, as postulated by Schopf<sup>2</sup>. This result supports a microplate nature of Western Antarctica.

The geology of the Ellsworth Mountains has been summarized by Craddock<sup>3</sup>. A continuous sedimentary sequence is estimated to be at least 13 km thick and to range in age from late Precambrian(?) to Permian. The outcrops display a variety of fold types with fold axis trending 310°E in the southern part to 345° in the north. The sedimentary section and style of folding have been compared with that in the Pensacola Mountains<sup>2,4</sup> and the Cape Fold Belt<sup>3</sup>, and the deformation in the Ellsworth and Pensacola Mountains is related to the early Mesozoic Gondwanide orogeny<sup>1,4</sup>.

The structural trends of the Ellsworth Mountains appear to be perpendicular to those of the Pensacola Mountains. Schopf<sup>2</sup>



**Table 1** Mean-site directions, statistics and pole positions from the Cambrian of the Ellsworth Mountains

| Site                                                                        | Sampling site |         | Tilt correction | N  | $\bar{D}$ | $\bar{I}$ | k  | $\alpha_{95}$ | Pole |       | dp   | dm    |
|-----------------------------------------------------------------------------|---------------|---------|-----------------|----|-----------|-----------|----|---------------|------|-------|------|-------|
|                                                                             | Lat.          | Long.   |                 |    |           |           |    |               | Lat. | Long. |      |       |
| 1                                                                           | 79°12'S       | 85°54'W | Before          | 7  | 331°      | 33°       | 14 | 17°           |      |       |      |       |
|                                                                             |               |         | After           |    | 13°       | 17°       | 15 | 16°           |      |       |      |       |
| 2                                                                           | 79°09'S       | 85°56'W | Before          | 8  | 328°      | 55°       | 6  | 26°           |      |       |      |       |
|                                                                             |               |         | After           |    | 20°       | 24°       | 6  | 26°           |      |       |      |       |
| 3                                                                           | 79°10'S       | 86°10'W | Before          | 8  | 14°       | 18°       | 22 | 12°           |      |       |      |       |
|                                                                             |               |         | After           |    | 20°       | 9°        | 25 | 11°           |      |       |      |       |
| 4                                                                           | 79°12'S       | 86°03'W | Before          | 8  | 352°      | -63°      | 6  | 24°           |      |       |      |       |
|                                                                             |               |         | After           |    | 36°       | 10°       | 6  | 24°           |      |       |      |       |
| 5                                                                           | 79°08'S       | 86°13'W | Before          | 10 | 22°       | -16°      | 11 | 15°           |      |       |      |       |
|                                                                             |               |         | After           |    | 21°       | 4°        | 11 | 15°           |      |       |      |       |
| Mean Upper Cambrian                                                         | 79°S          | 86°W    | Before          | 5  | 357°      | 8°        | 3  | 58°           | ***  | ****  |      |       |
|                                                                             |               |         | After           |    | 22°       | 13°       | 52 | 11°           | 4°N  | 296°E | 5.7° | 11.2° |
| Pole position after rotation 82° clockwise about a Euler pole (84°S, 300°E) |               |         |                 |    |           |           |    |               | 1°S  | 18°E  | 5.7° | 11.2° |

'Tilt correction' denotes whether the parameters are before or after structural correction is applied;  $N$ , number of samples or sites from which the mean is calculated;  $\bar{D}$ , mean declination;  $\bar{I}$ , mean inclination;  $k$ , estimate of Fisher's precision parameter<sup>17</sup>;  $\alpha_{95}$ , the apical half angle of the cone of 95% confidence<sup>17</sup>; the palaeomagnetic pole is calculated from the mean of the structurally corrected mean-site directions; dp and dm are the axes of the oval of 95% confidence<sup>18</sup>.

suggested that the Ellsworth Mountains are allochthonous with respect to the Pensacola Mountains and placed the Ellsworth Mountains north of the Pensacola Mountains (Fig. 1b) so that the structural trends align; this hypothesis has since been elaborated<sup>5-7</sup>. Others<sup>3,8</sup> have suggested that the Ellsworth Mountains are autochthonous, with the structural trends continuing beneath the ice, swinging through 90° to align with the Pensacola trend.

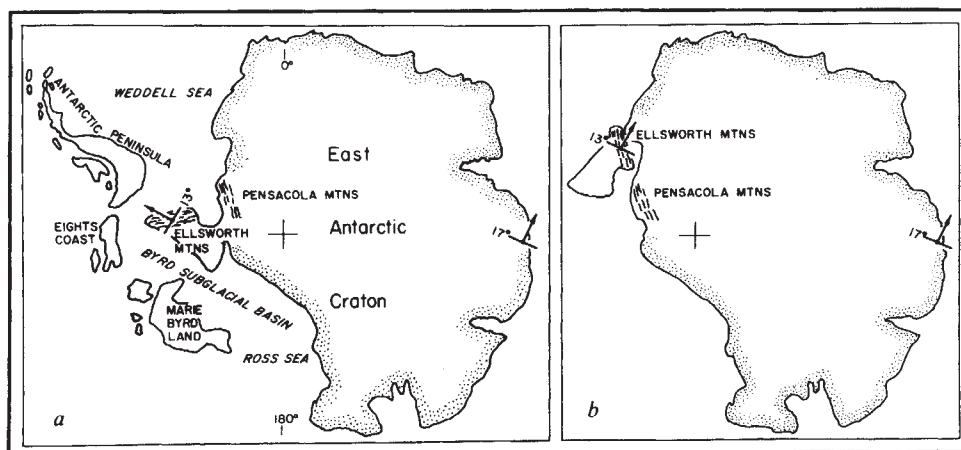
During the 1979-80 field season, a major field programme in the Ellsworth Mountains was conducted by the US Antarctic Research Program<sup>9</sup>, which included a systematic palaeomagnetic collection of the Cambrian to Devonian sedimentary section. Beforehand, the magnetic remanence of a suite of unoriented samples collected by expeditions during the early 1960s was investigated by stepwise alternating field and thermal demagnetization and plotting the results on the orthogonal projections of Zijderfeld<sup>10</sup>. Promising lithologies were identified on the basis of linear trajectories on the projections<sup>11</sup>, consistency of directions within a given sample and the physical properties of the remanence, such as coercivity and blocking temperature consistent with single-domain carriers. Haematitic argillites of the Upper Heritage Group from Pipe Peak (79°09'S, 84°14'W) were identified as promising targets because the highest blocking-temperature component of their multivector remanence had shallow inclination relative to the bedding plane. Palaeogeographical reconstructions<sup>12</sup> of Gondwanaland show Antarctica in an equatorial position only for the Lower Palaeozoic; the shallow inclination with respect to bedding identified for these argillites is therefore consistent with Lower Palaeozoic magnetization. They were therefore more extensively sampled over a wide area in 1979-80.

The stratigraphical horizons collected may be formally reclassified and renamed as a result of work done on this and previous expeditions (B. Spörl and G. Webers, personal communication). The sampling sites are stratigraphically within 200 m of a horizon traceable through certain structures in the northern Heritage Range. The horizon is dated to the Upper Cambrian on the basis of a trilobite fauna recovered on the flank of Pipe Peak during 1979-80 (G. Webers, personal communication). The sampling sites are spread over a 25-km<sup>2</sup> area. Sites 3 and 5 are in subhorizontal beds near the centre of the Pipe Peak Syncline, site 4 is on the vertically dipping, westward younging flank of this syncline, and sites 1 and 2 are in the vertically dipping eastward younging flank of the adjacent Tent Syncline.

Magnetic remanence was measured using the British prototype CCL cryogenic magnetometer and a DIGICO spinner magnetometer. Stepwise thermal demagnetizations were performed with a bench-type mu-metal shielded oven and a larger oven described in ref. 13. The results were analysed as described previously. Natural remanent magnetism (NRM) intensities range from 10<sup>-1</sup> Am<sup>-1</sup> to 10<sup>-4</sup> Am<sup>-1</sup> but are typically 10<sup>-3</sup> Am<sup>-1</sup>. Alternating field demagnetizations using a tumbler device resulted in only partial removal of secondary components and characteristic directions were not well resolved.

Table 1 summarizes the mean-site directions and associated statistics of the highest blocking-temperature components (650-670°C) revealed by thermal demagnetization. The mean is computed from the directions of magnetization isolated from individually oriented specimens. Figure 2 shows stereographical projections of the mean-site directions and cones of 95% confidence before and after structural correction. *In situ*

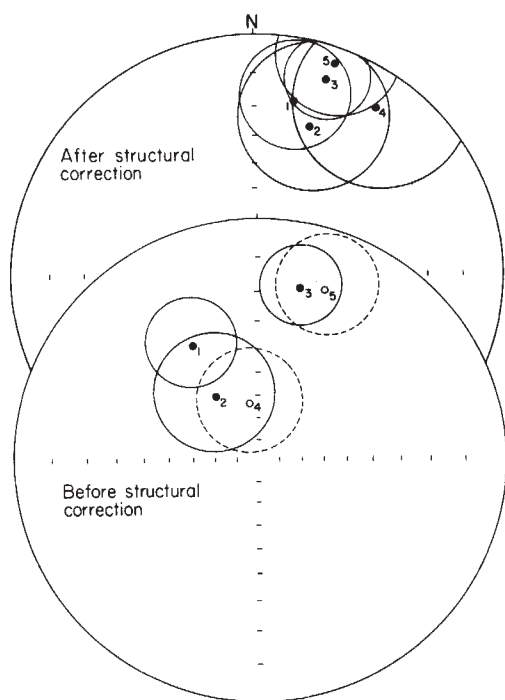
**Fig. 1** a, Map of Antarctica showing major blocks of continental crust above sea level after ice is removed<sup>1</sup>. The structural trends defined by fold axes are shown for the Ellsworth and Pensacola Mountains. Mean palaeomagnetic declinations and inclinations are indicated for the Cambrian argillites of the Ellsworth Mountains and the one late Cambrian result from the East Antarctic craton<sup>16</sup>. b, Map illustrating a suggested restoration of the Ellsworth Mountain block<sup>2</sup>. The mean palaeomagnetic declinations and inclinations illustrate the consistency of the present data with the rotation hypothesis. The extent of the Ellsworth block must be regarded as hypothetical.



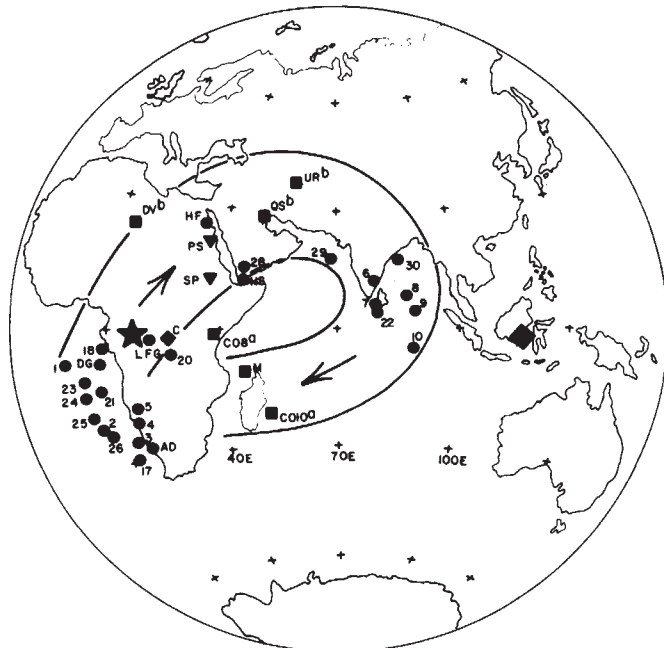
directions are different for each structural limb, and agree, with a complete overlap of the cones of confidence, only after the correction for the tilt of the beds is applied, proving a pre-tectonic age of the highest blocking-temperature component. Although the positive fold test does not prove a Cambrian acquisition of the highest blocking-temperature component, we still believe that the shallow inclination of the structurally corrected remanence does imply a Lower Palaeozoic age for this magnetization.

Studies of North American rocks indicate that the Upper Cambrian was a time of predominately reversed polarity<sup>14</sup>, although some normal polarity episodes are recorded in rocks from Australia<sup>15</sup>. We therefore believe that the components isolated from the Upper Cambrian argillites of the Ellsworth Mountains are probably of reversed polarity. Assuming this is the case, the palaeomagnetic south pole falls at 4°N, 296°E, approximately 70° of arc away from any known Gondwana early Palaeozoic palaeomagnetic south pole with respect to Antarctica. If the Ellsworth block is restored to a position like that advocated by Schopf<sup>2</sup> (Fig. 1b), the palaeomagnetic pole falls on Klootwijk's<sup>15</sup> interpretation of the Cambrian polar wander track (Fig. 3) between late middle Cambrian (poles 1, 18, 23 and so on) and Upper Cambrian (poles 28, 29) poles from Australia. The restored Ellsworth palaeomagnetic pole is also indistinguishable from the one late Cambrian pole available from the East Antarctic craton<sup>16</sup>. This consistency is depicted in Fig. 1, where the mean declinations of the palaeomagnetic results are compared before and after the restoration of the Ellsworth Mountains.

If the characteristic magnetizations from the Ellsworth Mountains are of normal polarity, the uncorrected south pole falls as indicated in Fig. 3, approximately 30° of arc away from the Gondwana polar wander track. This interpretation would still imply a rotation of the Ellsworth Mountain block, but in a sense opposite to that suggested by Schopf<sup>2</sup>, which would not align the structural trends.



**Fig. 2** Equal-angle stereographical projections of the mean-site directions with associated cones of 95% confidence, before and after structural correction. The mean directions are labelled as in Table 1. ●/○ Denote projections of the north-seeking polarity directions on the lower/upper hemisphere. The continuous/dashed circles denote the projections of the cones of confidence on the lower/upper hemisphere. The cones of confidence overlap only after the structural correction is applied.



**Fig. 3** Middle Cambrian to Cambro-Ordovician Gondwana polar wander path with respect to Antarctica after Klootwijk's<sup>15</sup>. Numbered poles are the Australian poles reported in ref. 15, (a) poles are from ref. 19, (b) poles are from ref. 20, all others are from ref. 21. Pole 'C' is the one late Cambrian result available from the East Antarctic craton<sup>16</sup>. The star denotes the location of the mean Upper Cambrian pole from the Ellsworth Mountains after a rotation suggested by Schopf<sup>2</sup> is applied (Table 1). The large diamond denotes the non-rotated south pole position assuming a normal polarity. ●, Australian poles; ◆, Antarctic poles; ■, African poles; ▼, Indian poles.

Uncertainties in polarity determination and the rapid polar wander with respect to Gondwanaland limit the usefulness of the present data in deriving a precise location of the Ellsworth Mountain block in Gondwanaland. However, assuming the characteristic magnetizations are of reversed polarity, the restoration suggested by Schopf<sup>2</sup> is consistent with these data. Our results support interpretations that Western Antarctica is composed of displaced terrains which have moved with respect to the major plates.

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## Evidence for a late Wisconsin glaciation of the Weddell Sea

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The huge ice shelves in West Antarctica—the Ross and Filchner/Ronne Ice Shelves—have probably extended out on the continental shelf during the late Wisconsin<sup>1</sup>. Previous discussions, which have focused on the Ross Sea, have suggested (1) that the ice extended across the whole continental shelf<sup>2,3</sup> or (2) that there was only a minor expansion<sup>4</sup>. Here we present sedimentological data from the Weddell Sea (Fig. 1) which suggest that a late Wisconsin grounded ice sheet extended to the shelf edge. The evidence includes a recent thicker ice in Ellsworth Mountains at the head of the Filchner/Ronne Ice Shelf<sup>5</sup>. This thickening would lead to an expansion of the inland ice sheet over the continental shelf, filling up the Weddell Sea embayment.

Geological fieldwork during the 1976–77 and 1978–79 Norwegian Antarctic Research Expeditions provided the following observations.

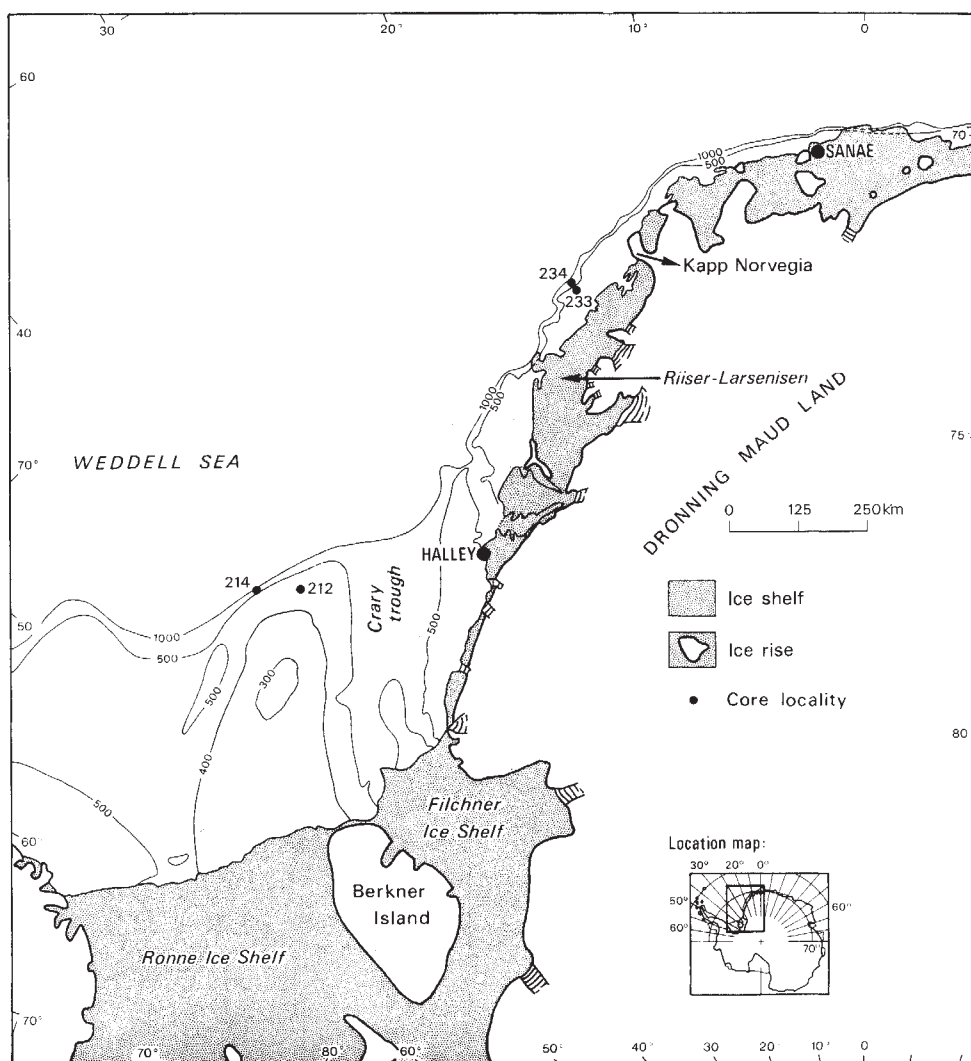
(1) Sediment samples (60 stations) show that the continental shelf is covered by stiff pebbly mud (till deposits), in places overlain by a thin veneer (<2 m) of soft pebbly mud (glaciomarine deposits). From the upper slope only glaciomarine sediments are recovered (Fig. 2). A similar lithology is described by Anderson *et al.*<sup>6</sup> (23 cores).

(2) Shallow seismic reflection (sparker) records show a veneer of unconsolidated sediments which rapidly thickens seawards off the shelf edge<sup>7</sup> (G. Maisy, personal communication).

(3) <sup>14</sup>C dating of shell fragments of the till, together with the age and distribution of the glaciomarine sediments, suggest a late Wisconsin age for the advance of a grounded glacier to a water depth<sup>8</sup> of 500 m.

The stiff pebbly mud is characterized by poor sorting, homogeneous texture and variable content of reworked fossils and significant amounts of Mg-calcite and cristobalite<sup>9</sup>. The degree of compaction—measured by cone—gave values in the range of 50–120 kN m<sup>-2</sup> on core samples from the shelf edge. Similar values have been recorded on late Wisconsin till on the Norwegian shelf<sup>10</sup>. The compaction of the stiff pebbly mud may have been caused by sediment loading. However, it is unlikely that submarine erosion has been able to remove completely a substantial overburden of glaciomarine-glacial deposits. In the latter case, a lag of coarser-sized material would be present, as observed in other glaciated shelf areas which have later been reworked subaqueously<sup>11</sup>. The values obtained for compaction show that the deposit is overconsolidated, which, in addition to

Fig. 1 Map showing the eastern part of the Weddell Sea and localities of cores shown in Fig. 2.



its textural composition and a broad areal distribution, can best be explained as being due to a grounded glacier.

Sediment thickness and distribution from shallow seismic reflection records also support the concept of grounded ice. Generally <30 m of unconsolidated, unstratified sediments are present on the continental shelf with frequent outcrops of the underlying bedrock. The thickness of unconsolidated sediments increases rapidly downslope from the shelf edge. Sediment distribution and texture in the Cray Trough also seem to indicate earlier glacial grounding. Overconsolidated deposits are present and in the inner and central parts bedrock is overlain by <20 m of sediments, while the bathymetric highs (200–300 m) excess elevation in the outer part of the trough are caused by a greater thickness of unconsolidated sediments forming a till–ridge complex.

A conclusion on the age of the till from a single  $^{14}\text{C}$  date is uncertain, especially when this age is >20,000 yr BP; however, a late Wisconsin age is supported by the following evidence. The coarser sand and gravel-sized carbonate particles were well preserved and showed no sign of dissolution. Furthermore, the probability for external carbon contamination<sup>12</sup> of the sample seems low: the exposure to seawater was followed by the reworking of a cold-based glacier<sup>8</sup> and encasement in clay-rich sediments.

Additionally, pre- and syn-late Wisconsin glaciomarine sediments are recovered on the upper slope, just outside the localities where till deposits are found (Fig. 2, Table 1). It is unlikely that the shelf edge should have been ice free for >40,000 yr with little or no glaciomarine deposition, while at least 2 m of sediments were deposited on the upper continental slope at localities within 20 km.

Sugden and Clapperton<sup>13</sup> argue against a large expanded late Wisconsin West Antarctic ice sheet as their observations from George VI Sound on the western coast of the Antarctic Peninsula revealed only a minor ice advancement. This does not, however, exclude the thickened West Antarctic ice sheet extending into the Weddell Sea embayment, but it does suggest that the different sections of the ice sheet margins may have moved out of phase.

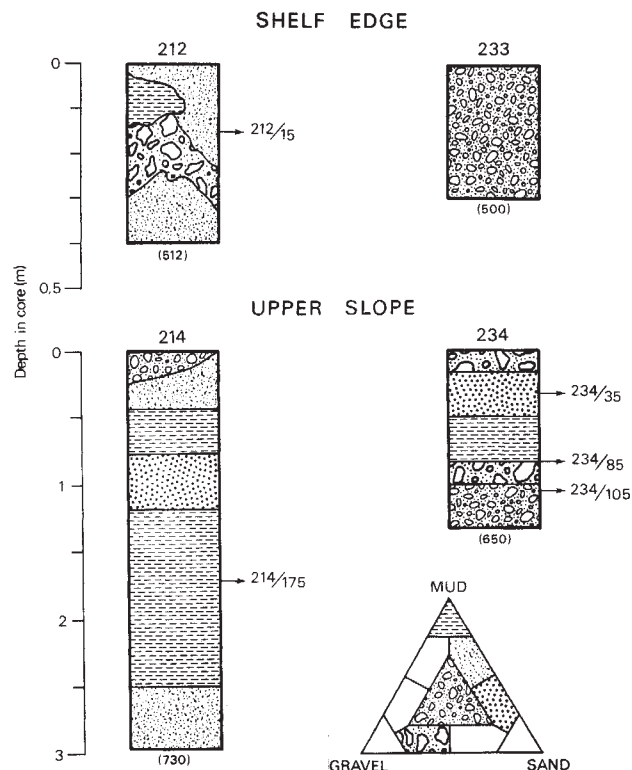


Fig. 2 Characteristic lithology of the sediments from the shelf edge and the upper continental slope in the southeastern Weddell Sea. The sediments recovered from the shelf edge are overconsolidated till deposits while the samples from the upper continental slope are glaciomarine. The numbers on the right side of the core refer to  $^{14}\text{C}$ -dated samples in Table 1. The numbers below the core show water depths.

Table 1 References for cores shown in Fig. 2

| Sample/<br>depth<br>in core<br>(cm) | Position                     | Water<br>depth<br>(m) | Type of<br>material                      | $^{14}\text{C}$ age                                                     |
|-------------------------------------|------------------------------|-----------------------|------------------------------------------|-------------------------------------------------------------------------|
| 212/15                              | S 74° 22.57'<br>W 37° 45.50' | 512                   | Shell<br>fragments<br>1–10 mm<br>in size | 31,290<br>1σ<br>+1,880<br>–1,520<br>2σ<br>+4,330<br>–2,800<br>(T–3,625) |
| 214/175                             | S 74° 06.15'<br>W 39° 26.44' | 730                   | Shell<br>bivalves<br>in situ             | >35,100<br>(1σ)<br>(T–3,835)                                            |
| 234/35                              | S 72° 10'<br>W 16° 35'       | 650                   | Bryozoan<br>debris                       | 21,240<br>±760<br>(T–3,617)                                             |
| 234/85                              | S 72° 10'<br>W 16° 35'       | 650                   | Bryozoan<br>debris                       | 28,130<br>±1,410<br>(T–3,618)                                           |
| 234/105                             | S 72° 10'<br>W 16° 35'       | 650                   | Bryozoan<br>debris                       | 37,830<br>±3,110<br>(T–3,332)                                           |
| 233                                 | S 72° 10.04'<br>W 16° 24.23' | 500                   |                                          |                                                                         |

Number in parentheses refers to sample number at Laboratoriet for Radiologisk Datering, Trondheim, Norway.

These data indicate that the Weddell Sea continental shelf was covered by grounded ice during the late Wisconsin. The Cray Trough also seems to have been covered by grounded ice, suggesting not only a simple areal expansion of the West Antarctic ice sheet, but also a considerable increase in thickness. The results support the concept of an expanded Antarctic ice sheet during the last great glaciation, as assumed in the CLIMAP reconstructions<sup>1</sup>.

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# High temperature of forest fires under pines as a selective advantage over oaks

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In many temperate ecosystems succession from pine forest to hardwoods is interrupted by fire, resulting in a fire climax dominated by pines<sup>1-3</sup>. As natural selection operates through both processes of succession and fire, early seral plants that are poor competitors may exhibit fire-facilitating characteristics whereas late seral plants that are superior competitors may show fire-retarding traits. Potential fire-retarding and fire-facilitating traits of plant foliage have been measured<sup>4-7</sup>, but their effects on survival or reproduction have never been demonstrated in the field<sup>7,8</sup>. We report here that maximum temperatures, recorded during fires in a mixed oak-pine woodland, were sufficiently higher under pines than under oaks to ensure elimination of the competitively superior oaks in the vicinity of adult pines.

Experimental fires were conducted in a Florida sandhill<sup>9</sup> where fire history had been recorded for six years. The area is dominated by longleaf pine (*Pinus palustris*), turkey oak (*Quercus laevis*) and sand live oak (*Quercus geminata*). The frequent surface fires are fuelled by dead grass (*Aristida spicata* and *Andropogon* species) and tree litter. The three tree species have different litters. Longleaf pine needles are 20–45 cm long and form a deep, well aerated layer directly under pine crowns. Turkey oak leaves are lobed and provide an aerated fuel where they lodge in grass blades, but these leaves are blown throughout the sandhill and are not concentrated under turkey oak crowns. Sand live oaks grow in groves that inhibit grass growth; their small elliptical leaves form a carpet of moist litter inside the groves. Previous work suggests that fire tolerance of the three species increases from live oak to turkey oak to longleaf pine<sup>10,11</sup>.

During three fires, maximum temperatures were recorded using 15 heat-sensitive waxes (Tempilstiks, Big Three Industries, South Plainfield, New Jersey) which melt at known temperatures, ranging from 52 to 538 °C (125 to 1,000 °F). The 15 waxes were marked on the surface of 2.5 × 7.0 cm ceramic chips, strung by thin wire at 0.5-m height intervals from 0.5 to 3.0 m above the soil. Six replicate wires were spaced at intervals of 0.5 m along 2.5-m transects in eight locations: three under longleaf pine crowns three under turkey oak crowns, and two in live oak groves. After the fire, two independent readings of the 288 chips estimated the maximum temperature (*T*) attained at each height and location.

Data analysis required a transformation of maximum temperature to maximum temperature increase ( $\Delta T$ ) above ambient, and logarithmic transformations of height and  $\Delta T$  to linearize the relationship between the two variables.

A multi-factorial nested analysis of variance in  $\Delta T$  showed significant variation for the two main factors (height and loca-

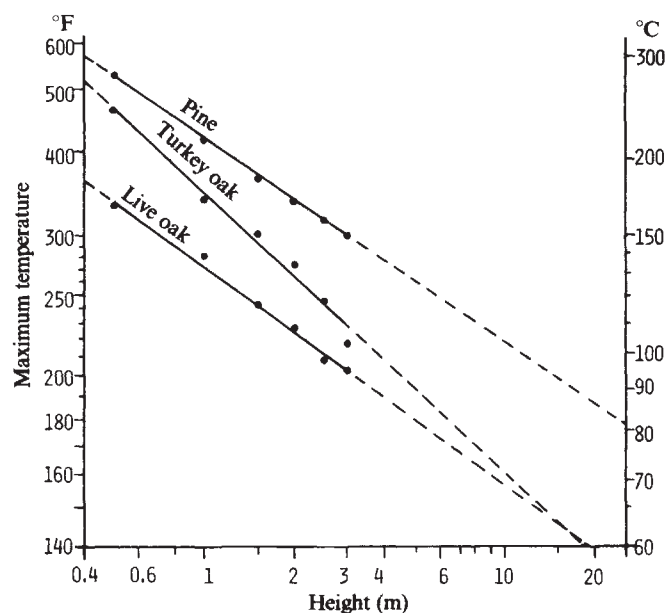


Fig. 1 Mean maximum temperatures, as a function of height above the litter surface, under crowns of longleaf pine (*Pinus palustris*), turkey oak (*Quercus laevis*) and sand live oak (*Quercus geminata*). Broken lines indicate projections of the regression lines beyond the heights of temperatures recorded.

tion) (*F*-test;  $P = 0.01$ ) but no interaction effect (*F*-test;  $P = 1.00$ ). Thus, maximum temperature increase was dependent on location under different species and on height above the litter, but maximum temperature increase declined with height in a manner common to all locations. Replicate wires (six per tree) showed no statistically significant variation (*F*-test;  $P = 0.61$ ), thus we conclude that there is little variation in  $\Delta T$  under any single tree. However, replicate trees showed significant differences (*F*-test;  $P = 0.01$ ), so there is an unhypothesized effect associated with variation among trees of the same species.

Further study of the main factors by analysis of covariance showed an inverse relationship of temperature on height that was significantly different ( $P < 0.001$ ) between each pair of locations. The linear regressions of the means of transformed  $\Delta T$  on transformed height are shown in Fig. 1 where the axes show the actual untransformed temperatures. For any given height the maximum temperature was greatest under longleaf pines and least in live oak groves. Two additional experimental fires in 1980 confirmed the relationships shown in Fig. 1. Differences in the amount of litter accumulated since earlier fires, as well as wind speed and moisture conditions at the time of each fire, resulted in a displacement of all three lines, but their relative positions remained unchanged.

Maximum fire temperature is critical to crown scorch and bud loss, which cause tree mortality<sup>12,13</sup>. Post-fire crown death from a large wildfire is illustrated in Table 1 for turkey oaks; taller oaks survived more frequently than shorter oaks (*G*-test;  $P < 0.005$ ), but location was as important as height—survival in full sunlight was twice as great as survival under longleaf pine crowns (*G*-test;  $P < 0.005$ ).

The results of our temperature measurements confirm that maximum fire temperatures are quite variable, as has been shown for fires in Scottish heath<sup>14</sup> and Nigerian savanna<sup>15</sup>. Models of fire intensity and behaviour are useful for comparisons among fires, but as noted by Albini<sup>16</sup>, the major source of error in such models is "that the fuel complex is not uniform, continuous, homogeneous, and consolidated into a single layer". Our results indicate that specific litter deposition patterns are a major cause of different intensities within fires. As these litter traits are subject to natural selection, different

Table 1 Per cent survival for each height class of turkey oaks, growing under longleaf pine crowns and in full sunlight

| Location            | Height class (m) |          |          |
|---------------------|------------------|----------|----------|
|                     | 1.0–1.9          | 2.9–2.9  | 3.0–10.0 |
| Under longleaf pine | 3% (34)*         | 10% (31) | 36% (36) |
| In full sunlight    | 6% (32)          | 18% (39) | 70% (64) |

\* Sample size in parentheses.

species may be regarded relatively as fire-facilitating or fire-retarding.

Apparent disasters, like fire, may be advantageous to some species relative to their interspecific competitors<sup>17</sup>. Pest outbreaks by generalized insects that consume several competing species may be advantageous to trees whose seeds and seedlings can dominate post-outbreak regeneration. In such a case, natural selection may have favoured herbivore-facilitating traits among some species when they reach a given age or size or when they face displacement by competitors. Where improved nutritional status of tree foliage is associated with pest outbreaks, as has been shown for balsam fir (*Abies balsamea*) and its pest, the spruce budworm (*Choristoneura fumiferana*)<sup>18</sup>, the outcome of the outbreak may be advantageous to the offspring of adult balsam fir trees when its white spruce (*Picea glauca*) competitors are destroyed as well as the firs, and the seedling balsam firs are thus released to grow in full sunlight<sup>17</sup>. Given such processes, then forest management must include the possibility that some tree species may facilitate the agents of destruction which are the targets of control.

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## Epidemic models used to explain biogeographical distribution limits

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It is an accepted principle in biogeography that the distributions of organisms are primarily controlled by the climate<sup>1–3</sup>; thus it is widely supposed that the geographical distribution limits of plants can be explained in terms of their physiological responses to particular climatic factors. However, such responses have rarely been demonstrated at natural distribution limits<sup>3</sup>; those responses that are reported generally have such small effects on survival that it is hard to explain how control could really be exercised by them. Those seeking explanations of distribution limits have, however, largely neglected the impermanence of the colonies constituting them. We suggest here that it is necessary to consider not only the effects of the climate on the performance of plants within existing sites but also the impact that these primary effects may have on the invasion of new sites. Epidemic models of the invasion process result in threshold theorems which explain how small changes in plant performance could result in large changes in the probability of new colonies appearing.

The difficulty in explaining distribution limits has been largely concealed by poorly founded statements about their cause<sup>2,3</sup>. Correlations between distribution limits and the isolines of synoptic climatic variables have been extensively described<sup>4–8</sup> but these cannot be attributed with causative significance<sup>3,9,10</sup>, and general studies on the responses of plants to climate have rarely focused on variations as small as those that occur across natural distribution limits<sup>7,11,12</sup>. Physiological explanations for the limits of a few perennial plants have nevertheless been proposed<sup>3,12</sup>. Surprisingly, failure to detect responses has been most marked among annual ruderals, which supposedly respond to the climate alone at their limits<sup>13</sup>. When introduced beyond their limits, many produce copious seed and persist locally. British examples which we have studied are *Lactuca serriola*, *Kickxia spuria* and *Lathyrus nissolia*; others are *Mercurialis annua*, *Kickxia elatine* and *Melampyrum arvense*<sup>3</sup>. Similar observations have been reported for sedentary marine animals<sup>14</sup>. Present concepts of distribution-limit control implicitly require that any limit-controlling responses to the climate—whether in establishment, growth or reproduction—should ultimately reduce fecundity so that continued regeneration is impossible. In the plants mentioned above, this does not seem to be the case, which leads us to believe that their limits are controlled by the small reductions in fecundity which may with difficulty be detected in some of them. However no mechanism for this has ever been proposed. Moreover many plants are abundant at their limits<sup>13</sup>, which are therefore abrupt relative to the climatic gradients that presumably control them, and this too is unexplained.

Little is known about the precise patterns shown by colonies of plants at their distribution limits but it is at least certain that they are constantly changing, even when the position of the limit is not. Distribution limits are abstractions from real patterns of colonies in the field and thus conceal their dynamic nature. It is therefore seldom noted that distribution limits might be controlled by climatic effects on the establishment of new colonies.

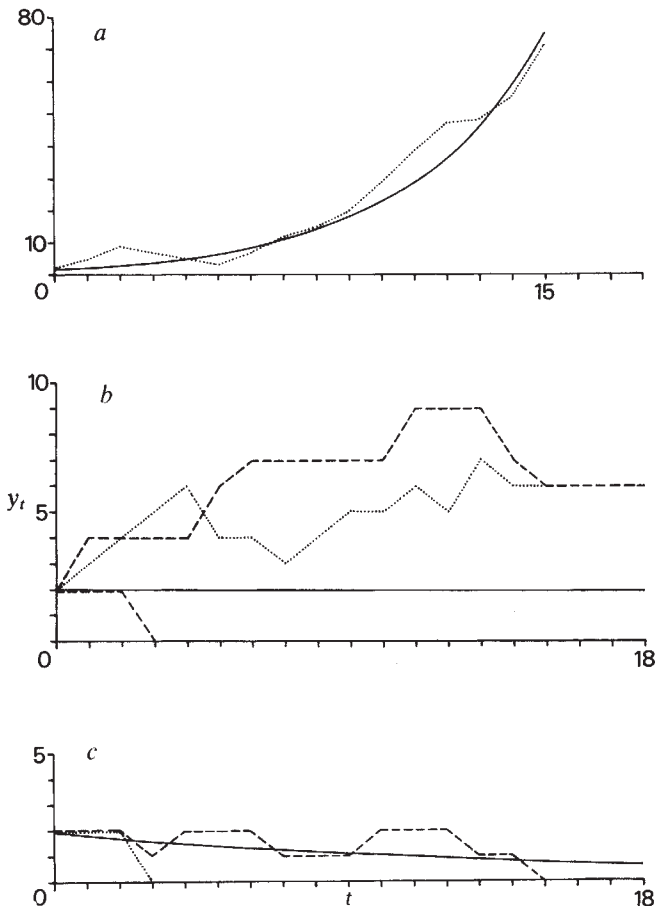
The spread of weeds has been likened to the spread of infectious diseases<sup>15,16</sup>, but the mathematical theory developed in connection with this<sup>17,18</sup> has only been used by botanists to describe the dispersal patterns of plant propagules<sup>16</sup> (although a related, population-dynamic model has been discussed<sup>19</sup>). There is, however, no reason why the general deterministic epidemic model<sup>17</sup>, which gives rise to the epidemic threshold theorem of Kermack and McKendrick<sup>17,20</sup>, should not be used to describe the infection of new sites.

We therefore define for a species the following:  $x$ , the number of susceptible sites in an area (unoccupied sites in which the species could grow);  $y$ , the number of infective sites (occupied sites from which it disseminates seeds);  $\beta$ , an infection rate dependent on the numbers of seeds produced in infective sites, efficiency of seed dispersal and the probability of establishment in susceptible sites; and  $\gamma$ , a removal rate dependent on the length of time for which infective sites remain occupied. Following the general deterministic epidemic model<sup>17</sup>, the rate at which susceptible sites become infective sites is given by

$$\frac{dy}{dt} = \beta xy - \gamma y$$

For the plant to spread,  $dy/dt$  must be positive, which requires  $x \geq \gamma/\beta$ . The relative removal rate  $\gamma/\beta$  therefore gives a threshold number of susceptible sites that must be exceeded if a small inoculum of the plant is to spread. Similar threshold theorems arise in epidemic models of all kinds, including stochastic and spatial ones<sup>18,21,22</sup>. These models may be applied to plants that occupy discrete sites and have observable rates of colonization and removal. They are largely valuable as strategic models (*sensu* May<sup>23</sup>) and their parameters may be difficult to estimate, although we believe this may be possible for some plants.





**Fig. 1** The population growth of a hypothetical plant simulated in an experiment which allowed control of infection rate ( $\beta$ ). The experiment was performed in a  $24 \times 8$  m area of cliff-top grassland at Peartree Point, South Devon. Susceptible sites for the plant were taken as those actually occupied by rosettes of *Plantago coronopus*. In each trial two rosettes were initially selected as occupied (infective) sites. From each, a specified number of coloured disks (mean diameter 9 cm) were thrown at random and rosette-centres on which disks landed were taken as being newly occupied sites. This procedure was carried out repeatedly, the total number of occupied sites being noted at each generation, that is, after each disk-throwing. Occupied sites were allowed to remain occupied for only three generations. Variation in the seed production of the plant was simulated by altering the number of disks thrown from each occupied site in different trials of the experiment. Broken lines show observed total numbers of occupied sites in successive generations for trials in which 10 (a), 5 (b) and 4 (c) disks were thrown from each occupied site. On completion of the trials the actual number of rosettes in the area was determined (number of occupied  $10 \times 10$  cm squares = 1,704). At generation  $t$ , the number of susceptible sites remaining ( $x_t$ ) is given by  $x_t = x_{t-1} - \beta w_{t-1} x_{t-1} + \gamma y_{t-1}$  where  $\beta$  is the probability of a particular disk landing on a particular rosette;  $w_{t-1}$  is the total number of disks thrown at generation  $t-1$ ;  $\gamma$  is the discrete-time removal rate for occupied sites; and  $y_{t-1}$  is the number of occupied sites at  $t-1$ .  $w_{t-1}$  can be rewritten as  $\lambda y_{t-1}$  where  $\lambda$  is the number of disks thrown from each occupied site. Similarly, the number of occupied sites at generation  $t$  is given by  $y_t = y_{t-1} + \beta \lambda y_{t-1} x_{t-1} - \gamma y_{t-1}$ . Non-extinction of the hypothetical plant requires  $y \geq y_{t-1}$  so that a threshold condition for its survival is given by  $x \geq \gamma/\lambda\beta$ . The discrete-time removal rate  $\gamma$  can be estimated as  $\gamma = 1 - e^{-\hat{\gamma}} = 0.2834$ , where  $\hat{\gamma}$  (continuous-time removal rate) is  $1/3$ . The infection rate  $\beta$  can be estimated from the area of disk relative to the total area ( $\beta = 1/30,181$ ). Substituting these estimates in  $x \geq \gamma/\lambda\beta$ , the threshold should have occurred at  $\sim \lambda = 5$ . Unbroken lines show expected values of  $y_t$  for successive generations. It can be seen that  $\lambda = 10$  gives a good fit to the expected curve;  $\lambda = 5$  seems to be about the threshold; extinction occurred in one trial and not in two. All trials with  $\lambda < 5$  led to extinction.

Along a climatic gradient, the number of susceptible sites for a plant could decline if its vegetational niche became less frequent; the infection rate could decline if its fecundity, dispersal or establishment were impaired; and the removal rate could increase if it were eliminated more quickly by vegetational

change. Within a plant's limit, the density of susceptible sites must exceed the relative removal rate. Towards the limit, changes in the climate might have small effects on the plant and its niche, which might increasingly alter the parameters of the threshold equation but the condition for spread ( $x \geq \beta/\gamma$ ) would continue to be satisfied. Ultimately, however, further small changes might so alter the parameters that this condition was not met, and there the plant would reach its limit. In this way climatic gradients could produce abrupt plant distribution limits even though the physiological responses elicited might appear too small to explain them. This idea is illustrated by an experiment (Fig. 1) in which we simulated the effect of reducing the seed output of a hypothetical plant. The results agree closely with the predictions of a discrete-time model as to the course of the plant's spread and the seed output necessary for its survival.

Most plants occur as casuals beyond their main areas of distribution and, to explain this, existing concepts of distribution-limit control require that casuals should only occur where conditions are especially favourable; but there is no evidence for this. In epidemic models susceptible sites clearly can exist beyond the limit, provided that their density nowhere exceeds the relative removal rate, and it is not surprising that plants should sometimes establish and perform well in them. It also follows that if the density of susceptible sites in an area near to but beyond a plant's distribution limit were artificially increased so as to exceed its relative removal rate, then the position of its climatic equilibrium distribution limit should change without any change in the climate or in the plant's biology. This has been deduced theoretically<sup>19</sup> and agrees with the observation that the sudden spread of some long-indigenous ruderals in Britain in the 1930s was related to the extensive building of arterial roads<sup>24</sup>. Another consideration is that as removal rates in perennials are obviously much lower than in annual ruderals, the density of susceptible sites required for their spread is therefore also lower, so that they should approach closer to the point where growth of the individual plant is prevented by climate. This would explain why responses to the climate should be harder to observe in annuals at their limits.

Although we have been concerned with the use of epidemic models to explain geographical distribution limits, they might also be used to explain small-scale limits, for example, those determined by local edaphic factors. The success of the simulation experiment (Fig. 1) reinforces this view.

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## Microheterogeneity of protein and sterol content in kidney podocyte membrane

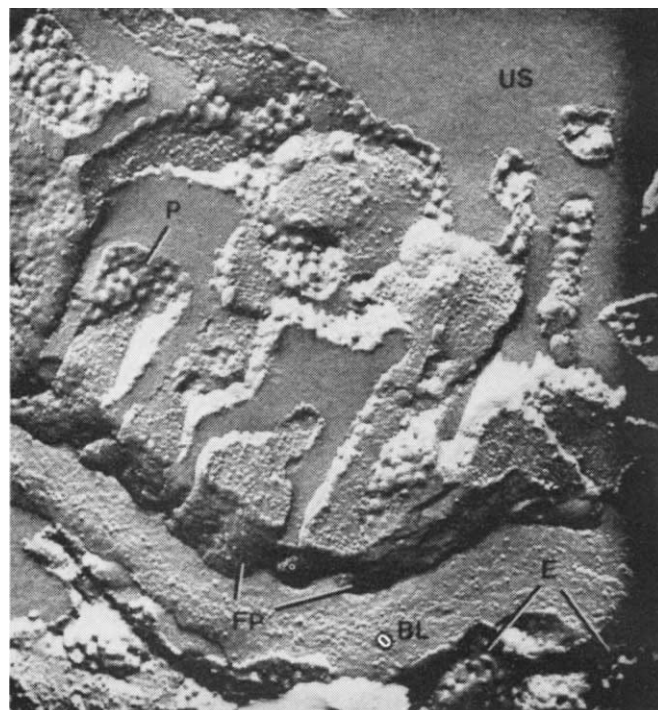
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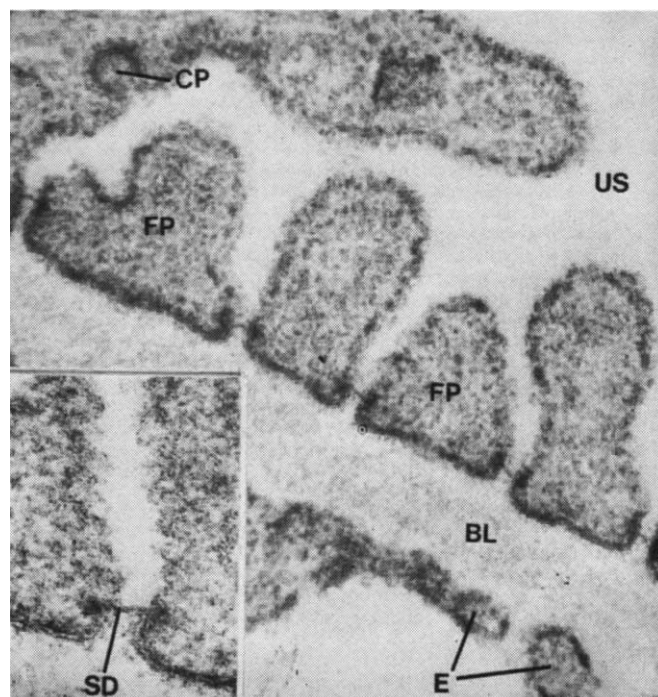
Podocytes are highly specialized epithelial cells which form part of the filtration barrier in the kidney glomerulus<sup>1</sup>. Their cell body sends out long 'foot processes' which interdigitate with those of neighbouring podocytes and which rest on the glomerular basal lamina. The maintenance of this characteristic cellular architecture is critical to the normal functioning of the kidney and may be controlled not only by cytoskeletal elements within the cell<sup>1,2</sup> but also by some unknown properties of the podocyte plasma membrane. Using freeze-fracture and a cytochemical probe for membrane cholesterol, filipin<sup>3,4</sup>, we show here that the protein content of and the number of filipin-sterol complexes in the podocyte plasma membrane are markedly reduced at sites of contact with the basal lamina, illustrating a precise zonal membrane differentiation in this region.

Adult male albino rat kidneys were fixed by perfusion through the aorta with 2.5% glutaraldehyde in 0.1 M phosphate buffer either with or without 300  $\mu$ M filipin (Upjohn), dissolved in 1% dimethyl sulphoxide. Kidneys were then removed and cut into  $\sim 15$ - $\mu$ m slices with a Vibratome (Oxford Instruments) to improve filipin penetration. The slices were incubated overnight at room temperature in a filipin-0.1 M cacodylate buffer solution. Glutaraldehyde fixation is necessary to prevent filipin-induced cell lysis and redistribution of intramembrane particles which occurs when non-fixed cells are exposed to the drug<sup>5</sup>. After rinsing in 0.1 M cacodylate buffer, the tissues were cryoprotected in 30% buffered glycerol, coated with polyvinyl alcohol<sup>6</sup> and frozen in Freon 22 cooled with liquid nitrogen. They were fractured and shadowed at  $-115^\circ\text{C}$  in a Balzers 301 freeze-fracture device<sup>7,8</sup>. Replicas were cleaned in sodium hypochlorite, rinsed in distilled water and recovered on copper grids before being examined in a Philips EM 300 electron microscope.

Podocytes are easily recognizable in freeze-fracture replicas by their position in the glomerulus and by the characteristic pattern of their foot processes<sup>8,9</sup> (Fig. 1). In addition, the part of the plasma membrane contacting the basal lamina (referred to in the text as the basal part of the foot process) can be distinguished from the other regions of the plasma membrane. In conventionally fixed material, the number and size of intramem-



**Fig. 1** Freeze-fracture of the glomerular filtration barrier of a filipin-treated kidney showing the plasma membrane of the capillary endothelium (E), the basal lamina (BL), podocyte foot process bases (FP), podocyte foot process legs (P) and the urinary space (US). Filipin-sterol complexes appear as stud-like protrusions or pits on all exposed membranes with the exception of the foot process bases.  $\times 50,000$ .



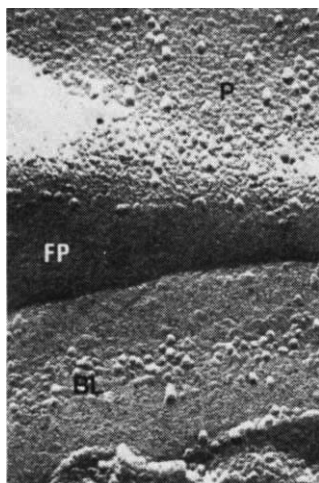
**Fig. 2** Thin section of a region of filipin-exposed kidney similar to that shown in Fig. 1. The typical trilaminar membrane structure is disrupted by filipin except where the foot processes contact the basal lamina. The inset shows that the membrane alteration occurs abruptly at the position of the slit diaphragm (SD) which links adjacent foot processes. The trilaminar membrane is clearly visible below the diaphragm whereas it is disrupted above this level. The trilaminar membrane structure is also retained at the level of a coated pit (CP) as previously reported<sup>15</sup>.  $\times 48,000$ . Inset:  $\times 113,000$ .

**Table 1** Quantification of intramembrane particles (IMP) and filipin-cholesterol (FC) complexes in podocyte plasma membrane (P-face)<sup>16</sup>

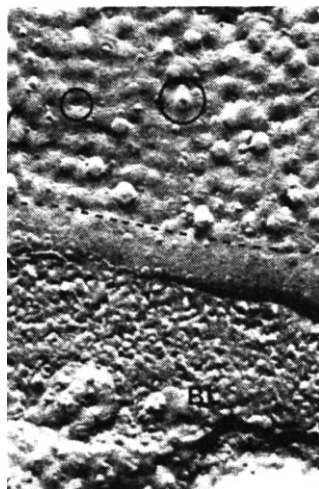
|                                                 | No. of IMP<br>(per $\mu\text{m}^2$ ) | Size of IMP<br>(nm)  | No. of FC<br>(per $\mu\text{m}^2$ ) |
|-------------------------------------------------|--------------------------------------|----------------------|-------------------------------------|
| Cell body and<br>legs (above<br>slit diaphragm) | $342 \pm 10$ (25)                    | $13.1 \pm 2.3$ (973) | $510 \pm 18$ (10)                   |
|                                                 | $P < 0.001$                          | $P < 0.001$          | $P < 0.001$                         |
| Foot process<br>bases (below<br>slit diaphragm) | $252 \pm 15$ (18)                    | $10.4 \pm 2.1$ (788) | $57 \pm 9$ (10)                     |

For IMP number, values in parentheses represent either the number of photographs analysed (for particle density) or the number of particles measured (for particle diameter) from at least seven animals. For FC number, the values in parentheses represent the number of glomeruli from five animals examined. Values are mean  $\pm$  s.e.m. Statistical analysis was performed using Student's *t*-test.

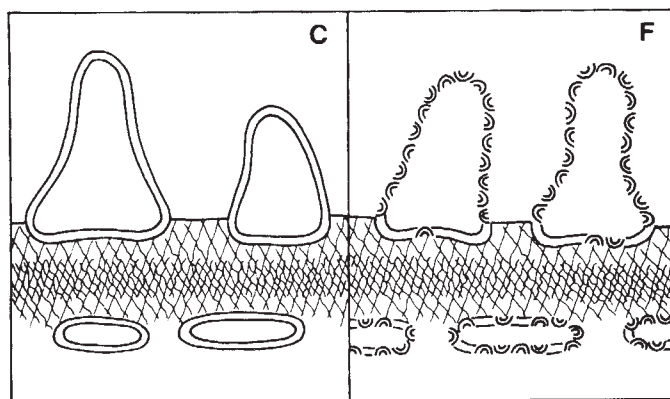




**Fig. 3** Higher magnification of a freeze-fractured podocyte foot process showing the sudden change in membrane particle content which occurs at the level of the slit pore diaphragm (represented by a discontinuous line of particles). BL, basal lamina; FP, foot process base; P, lateral membrane of foot process.  $\times 95,000$ .



**Fig. 4** Similar region to Fig. 3 but treated with filipin. An abrupt fall in the number of filipin-sterol complexes (small circle = pit; large circle = protuberance) occurs below the slit pore diaphragm (dashed line) where the foot process rests on the basal lamina (BL).  $\times 86,000$ .



**Fig. 5** Schematic representation of normal structure (C) and filipin-induced (F) membrane changes at the glomerular filtration barrier—compare with Fig. 2. The foot process membranes adjacent to the urinary space are extensively altered by filipin, whereas the basal portion which rests on the basal lamina contains relatively few filipin-sterol complexes. The entire membrane of the endothelial cell, however, has many complexes, including that portion in contact with the basal lamina.

brane particles in these membrane regions (Fig. 3) were quantified; Table 1 shows the results. The particles on the basal part of the foot processes are significantly smaller and less numerous than on the rest of the podocyte cell body. In the filipin-treated material, the regional differences were even more striking. Whereas the podocyte cell body and the 'legs' of the foot processes were heavily labelled with filipin-cholesterol complexes, the membrane adjacent to the basal lamina contained only a few complexes (Table 1, Figs 1, 4). In freeze-fracture replicas, filipin-cholesterol complexes appear as 25–30-nm stud-like protuberances or slightly smaller pits in the membrane face<sup>3</sup>, which are clearly different from the much smaller intramembrane particles. In suitable replicas, an abrupt reduction in filipin labelling could be seen on the membranes of podocyte foot processes as they reached the basal lamina (Fig. 4). This well-delimited frontier corresponds precisely to the position of the slit diaphragm, seen in thin sections, which connects the basal parts of adjacent foot processes. This can be appreciated in Fig. 2 which shows, in thin section, the alteration by filipin of the usual trilaminar membrane structure above the slit diaphragm, whilst the membrane below the slit diaphragm and adjacent to the basal lamina appears normal. Often, however, a small part of the plasma membrane above the slit diaphragm also appeared unmodified by filipin. In contrast to the poor labelling of the foot process bases, the entire plasma membrane of the capillary endothelium, including the region in contact with the basal lamina, showed abundant filipin-sterol complexes ( $417 \pm 22$ ,  $n = 10$ , see Table 1 legend) (Fig. 1). Furthermore, labelled vesicles were found in the cytoplasm of the foot processes, including in their basal portion. This indicates that the pattern of filipin labelling observed was probably not due to the uneven penetration of the drug. In fact, kidneys which were fixed by perfusion followed by injection of filipin into the renal artery, but which were not subsequently chopped and immersed in filipin, showed a similar pattern of labelling.

We have demonstrated that the plasma membrane of the basal part of foot processes differs from the podocyte cell body in terms of both the number of filipin-sterol complexes and its protein content (represented by intramembrane particles<sup>10,11</sup>). Figure 5 shows schematically the observed distribution of filipin-sterol complexes at the level of the filtration barrier. A similar scarcity of filipin-sterol complexes has also been found in the filopodia of fibroblasts at their point of attachment to the substratum<sup>12</sup>. The factors responsible for this local membrane heterogeneity are unknown, but the result is the formation of highly differentiated membrane domains which, in the present case, may be important in maintaining specific architectural relationships critical to the normal functioning of the kidney filtration barrier. The importance of the structural integrity of the barrier components is underlined by the loss of podocyte foot processes in glomerular nephrotic syndrome<sup>9,13,14</sup>.

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## Limitations of cell kinetics in distinguishing cell cycle models

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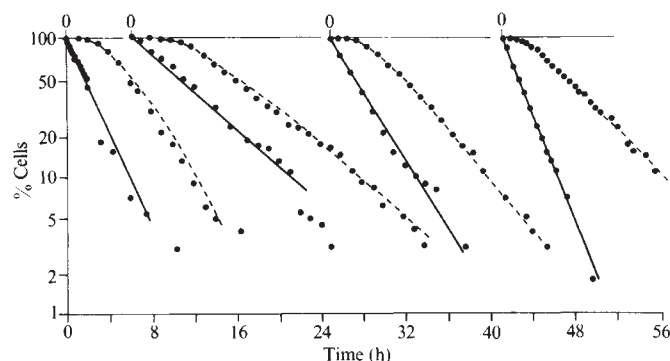
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There has recently been considerable interest in the variability observed in the rate of transit of mammalian cells through the cell cycle. Much of the discussion has centred around the transition probability model<sup>1,2</sup>, partly because the kinetics are easily analysed, but largely because it proposes a single event which controls the rate of cell division. To find such an event would be of importance in the study of control of the rate of growth of mammalian cells. Here, we examine several mathematical models for analysing cell cycle kinetic data, and discuss some of the problems of this approach.

It should be stated at the outset that from a mathematical point of view, for any given set of data there is an infinite set of mathematical approximations to describe it. The position is never reached where only a single formulation applies to real data. Consequently, although a mechanism may be ruled out by the kinetics, it cannot be proved by them; kinetic observations are at best suggestive of mechanism.

Analysis of kinetic data has usually been performed using  $\alpha$  and  $\beta$  curves; the  $\alpha$  curve is a plot of  $\alpha_t$  (the fraction of cells with generation times  $\geq t$ ) against  $t$ , the  $\beta$  curve is a plot of  $\beta_t$  (the fraction of sibling pairs with generation times differing by times  $\geq t$ ) against  $t$ . Because the original transition probability model predicts exponential  $\alpha$  and  $\beta$  curves, it is convenient to plot the  $\alpha$  and  $\beta$  curves on semi-log paper, to obtain linearity. The  $\alpha$  and  $\beta$  observed for exponentially dividing cell populations are of the form shown in Fig. 1.

We have examined mathematical models to see how the  $\alpha$  and  $\beta$  curves they generate compare with the data in Fig. 1. All models have been adjusted to have the same median and similar means and standard deviations, and were generated on computer using a Monte Carlo program. As predicted, the simple transition probability model yields completely linear and parallel  $\alpha$  and  $\beta$  curves (Fig. 2a; curve for  $n = 1$ ). Because  $\alpha$  curves are nonlinear, sources of variability must be added to the  $\alpha$  curve in addition to those determining the  $\beta$  curve. This may change the parallel relationship between the  $\alpha$  and  $\beta$  curves as



**Fig. 1**  $\alpha$  (---) and  $\beta$  (—) curves for (from left to right) 3T3 cells, L cells and two populations of CHO cells. The data are from ref. 8, replotted with a logarithmic y-axis. The scale on the lower abscissa refers to the  $\beta$  curve for the 3T3 cells. The  $\beta$  curves for the other cell populations have been displaced as indicated on the upper abscissa. The  $\alpha$  curves have been arbitrarily displaced so that the slopes can be compared with the slope of the corresponding  $\beta$  curve.

discussed below. The model can be extended to cover a requirement for  $n$  stochastic events. As  $n$  increases, the upper parts of the  $\alpha$  and  $\beta$  curves become curved, while the lower parts remain straight and parallel. As  $n \rightarrow \infty$ , the distribution of cell cycle times tends to become normally distributed. Shields<sup>3</sup> observed that neither the normal distribution nor the reciprocal or log normal distributions would generate linear  $\beta$  curves. It was concluded, therefore, that observations of a linear  $\beta$  curve would limit the model to the exponential one, or a trivial variant of it<sup>3,4</sup>.

The reciprocal or log normal distributions contain, in addition to the mean and standard deviations, a third parameter, the threshold<sup>5</sup>. This is the point mapped to  $+\infty$  on inversion or  $-\infty$  on taking logarithms. It is implicit in Shields' paper<sup>3</sup>, and explicit in Pardee's<sup>5</sup>, that the threshold of the transformation, whether inverse or log, should be at the start of the cell cycle. As the mammalian cell cycle is divided into phases, this seems to be an unnecessary assumption<sup>6</sup> but, if followed, leads to a distribution scarcely distinguishable from the normal distribution itself. A more skewed transformation may be obtained by bringing the threshold ( $Z$ ) closer to the mean ( $M$ ). In specifying an inverse or log normal generator it is convenient if the mean and standard deviation are not much changed as the threshold is varied. If  $G$  is the normal stochastic generator with mean  $M$  and standard deviation  $\sigma$ , a suitable log normal generator is

$$L(Z) = Z + (M - Z) \exp[(G - M)/(M - Z)] \quad (1)$$

By varying  $Z$ , it is possible to generate  $\alpha$  and  $\beta$  curves that are approximately linear, or concave upwards or downwards depending on the value of  $Z$ .  $\beta$  curves produced from  $L(Z)$  with  $M = 100$  and  $\sigma = 20$  are shown in Fig. 2b.

Similarly, an inverse normal generator of the form

$$I(Z) = Z + (M - Z)^2/(G - Z) \quad (2)$$

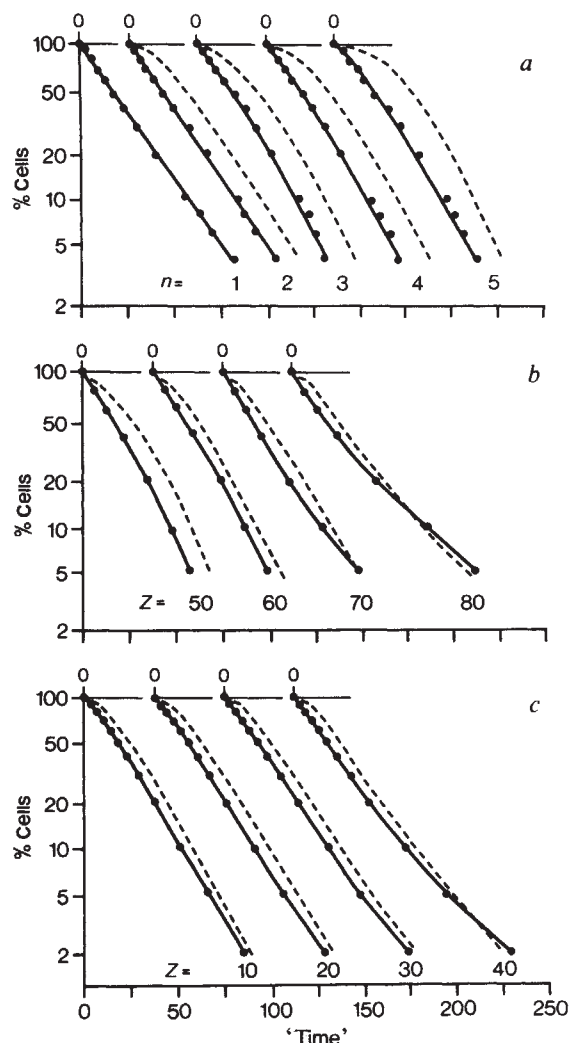
yielded the curves in Fig. 2c.

Note that where they are linear, the  $\alpha$  and  $\beta$  curves are also parallel. Both the transition probability model and the 'G<sub>1</sub> rate normal' model need modification if they are to include those cell populations in which the  $\alpha$  and  $\beta$  curves are clearly not parallel<sup>7,8</sup>. This was done by adding a generator to represent variability in the population which is shared by sibling pairs. The 'Bt' curves of Brooks *et al.*<sup>8</sup>, that are essentially linear on semi-log plots with a shoulder for the top 20% of values, may be obtained from any positively skewed leptokurtic ('pointed') generator. That an additional generator is required is also demonstrated by Shields's<sup>3</sup> observation that cell pairs chosen at random showed a different  $\beta$  curve from one based on sibling relationship.

Shields<sup>2</sup> also examined  $\beta$  curves for groups of pairs selected on the basis of minimum cycle time within the pair, and found linearity and parallelism for his cell population. However, this is a property not only of exponential curves<sup>3</sup>, but also of reciprocal ('rate') normal<sup>7</sup> and log normal curves (Fig. 3). If the population is heterogeneous<sup>6</sup>, such parallelism between groups would not be expected because selection of sibling pairs would then select lines with different cycle time variability. This would rule out many alternative explanations of  $\beta$  curve linearity based on heterogeneity<sup>6,9</sup> propagated from one cell generation to the next should the results prove to be reproducible on a large sample.

Accepting that data can be fitted by a variety of curves, we may ask how many data points are required to distinguish between the basic models. In the absence of real data on large numbers of cells the question has been formalized as follows: how many cells need to be observed before a particular model can be shown to generate a significantly nonlinear  $\beta$  curve (the shapes of the  $\alpha$  curves are easily described by a number of models<sup>6</sup>)? The series of curves in Fig. 2a-c show that outside a limited range of  $n$  or  $Z$  the  $\beta$  curves can be readily distinguished from the curves generated by the transition probability (exponential) model. Within the range, discrimination is more difficult as random fluctuations from linearity would also be expected even if the exponential model is used. The range of





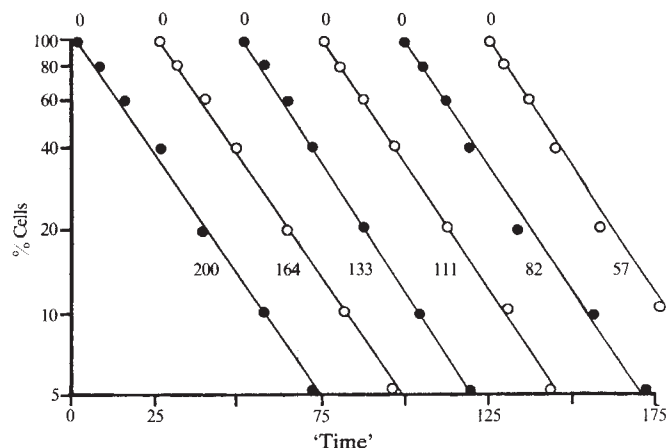
**Fig. 2** *a*, Monte Carlo simulations of  $\alpha$  (---) and  $\beta$  (—) curves for the transition probability model, and variants of it where  $n$  random events are assumed to be required before cell division. The simulation used 500 sibling pairs. The points on the  $\beta$  curves are computer generated values at representative percentiles so as to indicate the stochastic variability. For  $n = 1$  the  $\alpha$  and  $\beta$  curves are coincident. The origins of the curves have been displaced along the abscissa as in Fig. 1. *b*, Monte Carlo simulations of  $\alpha$  (---) and  $\beta$  (—) curves for a log normal model according to equation (1), with values of  $Z$  as indicated on the curves. The simulation used 200 sibling pairs. *c*, Monte Carlo simulations of  $\alpha$  (---) and  $\beta$  (—) curves for an inverse normal model according to equation (2). The simulation used 500 sibling pairs.

acceptance as exponential is wider if the number of data points per trial is smaller; to obtain an objective assessment of the curves it is necessary to select an efficient statistical test and also a probability level for rejection of the exponential model if it is true (type I errors). We have no reason to specify a single type of alternative model and have used the two-sided Kolmogorov test<sup>10</sup>, which is more powerful than the  $\chi^2$  goodness of fit test<sup>11</sup>. The time constant required to specify the exponential model was calculated for each trial by averaging the sibling time differences in the sample defining the  $\beta$  curve<sup>11</sup>. The probability of type I errors was set at 0.02 using limits derived from 1,000 trials of the exponential generator. The alternative models were then tested to determine (in 50 trials) the frequency of type II errors, that is, acceptance as exponential when the exponential hypothesis is not true. Obviously, changing the time constant would alter the fit of the data. For some of the inverse normal curves, a better fit is obtained if the last 5 or 10% of the data points (those with great 'sibling differences') are not used in deriving the time constant. The procedure removes the points that arise when  $G$

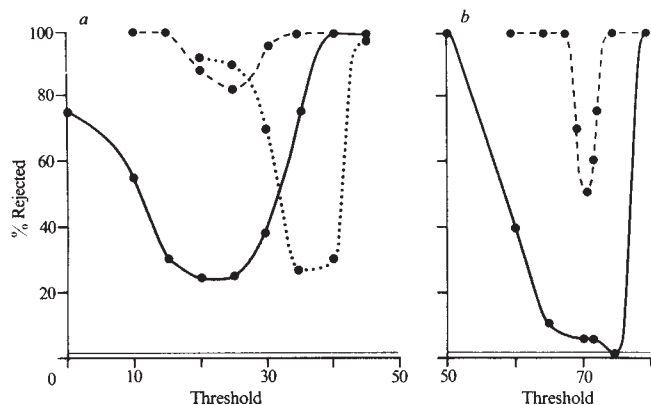
of equation (2) becomes nearly equal to  $Z$ . The procedure has no significant effect on the curve fitting if the hypothesis that the generator is exponential is true. In experimental situations, such points of the  $\beta$  curves are likely to be the most doubtful, or even unavailable if the experiment is of limited duration.

Then, if sibling pairs from 1,000 'cells' are used for each curve, a two-event transition probability model is rejected as exponential on 50% of occasions, while complete rejection is achieved if 4,000 cells are used. The inverse normal is completely rejected as exponential at 4,000 cells, unless the time constant is derived from the first 95% of observations, when 10,000 cells are required to rule out the model. The best fit of the inverse normal model is obtained with a threshold of 25 if all data points are used for derivation of the time constant, or 35 if 95% of points are used (Fig. 4). With the log normal model, 10,000 cells are sufficient to reject as exponential all but a narrow range of thresholds between 70 and 73, where the rejection rate falls to 50%.

Computer-generated data based on alternative models can be essentially discriminated at the 10,000 cell level. However, in considering the application of models to experimental situations, it is pertinent to ask how much variation in the time constant of the exponential or in the so-called determined part of the cell cycle would be needed to cause rejection of the  $\beta$  curve of a simple exponential model. In fact, with 10,000 cells, a 50% rejection as exponential of the exponential model is obtained by a normally distributed variation (s.d.) of  $\pm 20\%$  in the time constant of the exponential, or of  $\pm 16\%$  in the 'determined' part of the cycle relative to the exponential time constant. With the population A (3T3 cells) of ref. 8 as an example, the mean inter-mitotic time is approximately 10 times the exponential time constant derived from the  $\beta$  curve. Therefore, relative to the cell cycle as a whole, the variations would be only  $\pm 2\%$  or less and are modest by biological standards. Regardless, then, of the extent to which the real  $\beta$  curves retain linearity on further investigation, it seems unlikely that further kinetic studies or changes in the statistical methods used to analyse the data will in fact differentiate between the various models. Variation in the determined part of the exponential model leads to a shoulder at the top of the  $\beta$  curve but variations in the time constant lead to curvature at the tail. All the models discussed here can, therefore, be adapted to any experimental data which may show either a shoulder in the  $\beta$  curve or a curvature in its tail.



**Fig. 3** Monte Carlo simulation of the log normal model as equation (1) with  $Z = 60$ :  $\beta$  plots for 200 pairs and for pairs selected on the basis of a minimum cycle time requirement within the pair. Numbers on the curves give the number of selected pairs. The origins of the curves are again displaced as indicated. In the small samples the alternative method of dividing the sample into independent sub-groups<sup>3</sup> resulted in inconsistent relationships between the  $\beta$  curves of the sub-groups because of statistical fluctuations. With both this model and the transition probability model, different trials showed differing relationships between  $\beta$  curves of the sub-groups.



**Fig. 4** Rejection of inverse normal (*a*) and log normal (*b*)  $\beta$  plots by the Kolmogorov test. *a*, Pairs derived from 1,000 (—) and 4,000 (---) data points were used. The dotted curve shows decreased rejection of the data with 4,000 data points when the time constant for the comparative exponential is derived from the first 95% of observations only. Complete rejection was found with 10,000 data points (see text). *b*, Pairs derived from 1,000 (—) and 10,000 (---) data points were used. The line drawn parallel to the abscissa represents the extent of rejection of  $\beta$  plots of the single event exponential model.

Although the rate normal and the transition probability models have been used to propose simple but different mechanisms for inducing variability of cell cycle times, a variety of additional mechanisms may produce an exact<sup>12</sup> or approximate<sup>13</sup> log normal distribution. Attempts to deduce mechanisms consistent with the kinetic data are, therefore, pointless, particularly because any of the models can easily be adjusted to accept new data.

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## Direct inhibition of testicular androgen biosynthesis revealing antagonadal activity of neurohypophysial hormones

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Vertebrate neurohypophysial hormones are known to have antidiuretic, vasopressor and oxytocic properties<sup>1,2</sup>. However, arginine-vasotocin (AVT), the most primitive of the neurohypophysial principles and one that has been reported to occur in the mammalian pineal gland<sup>3–7</sup>, also retards murine pubertal progression<sup>8–10</sup> and canine testicular steroidogenesis<sup>11,12</sup>. Although AVT may exert its inhibitory effect *in vivo* by reducing the release of pituitary gonadotropins<sup>13,14</sup>, the possibility of a direct antagonadal action cannot be ruled out. Using a recently

established primary culture system of rat testicular cells<sup>15</sup>, we now report that AVT as well as other naturally occurring neurohypophysial hormones can act independently of the hypothalamic–pituitary unit to exert a direct inhibition of testicular androgen biosynthesis *in vitro*. The observation that this inhibitory activity can be blocked by vasopressor-selective, but not oxytocic-selective antagonists, suggests that the antagonadal action of the neurohypophysial hormones is probably mediated by highly stereospecific testicular receptor sites. These findings constitute the first demonstration of a direct antagonadal activity of neurohypophysial hormones and may account, in part, for the previously recognized antireproductive activity of AVT. Future design of synthetic analogues of neurohypophysial hormones may lead to the development of new male contraceptive peptides capable of directly inhibiting Leydig cell androgen biosynthesis.

The effects of nine naturally occurring neurohypophysial hormones on the human chorionic gonadotropin (HCG)-stimulated secretion of testosterone into the medium was investigated *in vitro*. Treatment with HCG (10 ng ml<sup>-1</sup>) led to a 68-fold increase in the secretion of testosterone compared with untreated controls (Fig. 1). Concomitant treatment with increasing concentrations (10<sup>-11</sup>–10<sup>-6</sup> M) of various neurohypophysial hormones resulted in a dose-dependent inhibition of the HCG-stimulated testosterone secretion. Arginine-vasopressin (AVP), AVT and lysine-vasopressin (LVP) proved most potent, with a projected minimal effective concentration of ~10<sup>-10</sup> M and median effective doses (ED<sub>50</sub>s) of 2.5 ± 0.2, 2.8 ± 0.1 and 5.1 ± 0.3 × 10<sup>-10</sup> M, respectively. (The projected minimal effective concentration is defined as the concentration leading to a decrease of 2 s.d. of the HCG-stimulated production of testosterone. Dose–response curve fitting and median effective dose (ED<sub>50</sub>) determinations were carried out by a computer program based on a four-parameter logistic equation<sup>16</sup>). As a group, these three peptides were about 100-fold more potent than mesotocin, valitocin and oxytocin (ED<sub>50</sub> 3.6 ± 0.2, 5.6 ± 0.4 and 7.1 ± 0.4 × 10<sup>-8</sup> M, respectively). In contrast, 10<sup>-6</sup> M of isotocin, glutitocin and aspartocin were virtually without effect. Pressinoic acid, the cyclic pentapeptide constituting the ring structure of AVP and LVP<sup>17</sup>, was also without effect (data not shown). As shown in Fig. 2, a significant (*P* < 0.01) positive correlation was found between the antagonadal potencies of the neurohypophysial peptides and their known antidiuretic (*r* = +0.96) and vasopressor (*r* = +0.97)

**Table 1** Effect of treatment with vasopressor- and oxytocic-selective antagonists on the AVT-induced inhibition of androgen biosynthesis

| Treatment                                                  | Testosterone secretion (ng per culture) |
|------------------------------------------------------------|-----------------------------------------|
| None                                                       | 0.04 ± 0.01                             |
| HCG                                                        | 2.78 ± 0.08                             |
| HCG + AVT                                                  | 1.30 ± 0.10‡                            |
| HCG + GnRH                                                 | 1.73 ± 0.15‡                            |
| HCG + AVT + d(CH <sub>2</sub> ) <sub>5</sub> -Tyr-(Me)AVP* | 2.71 ± 0.10                             |
| HCG + AVT + d(CH <sub>2</sub> ) <sub>5</sub> -TOT†         | 1.32 ± 0.05‡                            |
| HCG + GnRH + GnRH antagonist                               | 2.61 ± 0.17                             |
| HCG + AVT + GnRH antagonist                                | 1.28 ± 0.13‡                            |

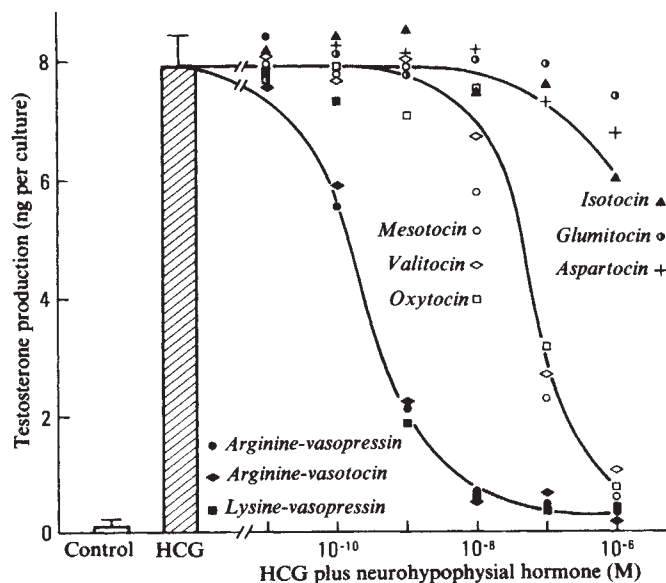
Testicular cells were cultured as described in Fig. 1 legend for 3 days in the presence or absence of HCG (10 ng ml<sup>-1</sup>), AVT (10<sup>-9</sup> M), GnRH (10<sup>-8</sup> M), a vasopressor-selective antagonist [d(CH<sub>2</sub>)<sub>5</sub>-Tyr-(Me)AVP] (10<sup>-6</sup> M), an oxytocic-selective antagonist [d(CH<sub>2</sub>)<sub>5</sub>-TOT] (10<sup>-6</sup> M), a GnRH antagonist (Ac-D-Phe<sup>1</sup>-D-pCl-Phe<sup>2</sup>-D-Trp<sup>3,6</sup>-GnRH) (10<sup>-6</sup> M), or appropriate combinations thereof. Medium testosterone content was measured by radioimmunoassay. The results are expressed as the mean ± s.e. of quadruplicate cultures.

\* [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),2-(O-methyl)tyrosine]arginine-vasopressin.

† [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),4-threonine]oxytocin.

‡ Significantly different (*P* < 0.05) from cultures treated with HCG alone.





**Fig. 1** Effect of nine naturally occurring neurohypophysial hormones on the HCG-stimulated secretion of testosterone. Testicular cells were obtained from adult (50–70 days old) hypophysectomized male rats and cultured without treatment for 8 days during which time media were replaced every 2 days<sup>15</sup>. The cells were then cultured for 3 additional days in the presence or absence of  $10 \text{ ng ml}^{-1}$  of highly purified HCG (Canfield CR-121, 13,450 IU per mg) and increasing concentrations ( $10^{-11}$ – $10^{-6}$  M) of various neurohypophysial hormones. The present culture constitutes a whole testicular dispersate which is more responsive to gonadotropins than isolated Leydig cells and is believed better to approximate the physiological conditions. Medium testosterone content was measured by radioimmunoassay. Neurohypophysial hormones did not interfere with the testosterone radioimmunoassay (data not shown). Measurements were further validated by the demonstration of comparable testosterone levels in ether-extracted samples. The results are expressed as the mean of quadruplicate cultures. Dose-response curves are provided for three representative peptides: arginine-vasopressin, oxytocin and isotocin. Curve fitting used the four-parameter logistic equation<sup>16</sup>.

potencies<sup>18</sup>. In contrast, no significant correlation was found between the antagonodal and oxytocic potencies<sup>18</sup> of these peptides ( $r = -0.24$ ;  $P > 0.1$ ). Also, treatment with AVP, AVT, LVP or oxytocin did not interfere with the binding of HCG to its receptors (data not shown) and the antagonodal effect of the neurohypophysial hormones was partially reversible 5 days after removal of the peptides (data not shown). The inhibitory effect of the neurohypophysial peptides was not associated with alterations in the total cell number, cell viability, cellular DNA or protein content (data not shown) and became statistically significant ( $P < 0.05$ ) after 12 h of treatment. Furthermore, the inhibitory effect of AVT on androgen biosynthesis was effectively blocked by vasopressor-selective<sup>19</sup> but not oxytocic-selective<sup>20</sup> antagonistic analogues of neurohypophysial hormones (Table 1). A potent antagonist of gonadotropin releasing hormone (GnRH), Ac-D-Phe<sup>1</sup>-D-pCl-Phe<sup>2</sup>-D-Trp<sup>3,6</sup>-GnRH, capable of antagonizing the antagonodal activity of GnRH, was without effect on the AVT-induced inhibition of androgen biosynthesis (Table 1).

AVT or AVT-like activity has been reported in the pineal of several mammalian species<sup>3–6</sup>, including man<sup>7</sup>. As several recent reports suggest the presence of AVP-like immunoreactivity in the pineal gland<sup>21,22</sup>, our observations raise the possibility that AVP also constitutes part of the antagonodal activity of pineal extracts. Taken together, our findings extend the range of biological activities ascribed to the neurohypophysial hormones to include a direct antagonodal effect.

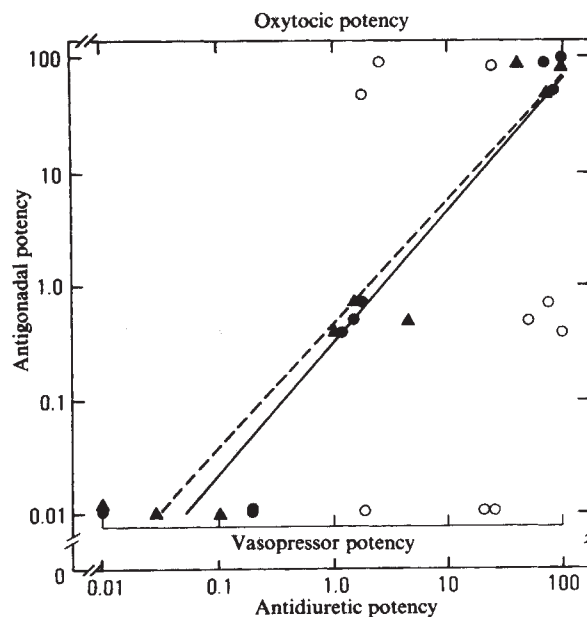
The reported circulating concentrations of neurohypophysial hormones in various adult mammals are of the order of  $10^{-12}$  M

(refs 23–25). Consequently, the antagonodal effects of neurohypophysial hormones reported here (minimal effective dose  $10^{-10}$  M) may be considered pharmacological. However, one is tempted to speculate that the antagonodal property of neurohypophysial hormones represents a primitive physiological function in lower vertebrates that was lost during evolution. Analogous reasoning can be applied to the vasopressor and vasodepressor functions of neurohypophysial hormones. Also, the neurohypophysial hormones or closely related peptides may be produced within the gonad, yielding local concentrations high enough to inhibit certain gonadal functions.

The mechanisms whereby neurohypophysial hormones inhibit testicular androgen biosynthesis remain unknown. However, the striking correlation between the antagonodal and the antidiuretic and vasopressor potencies of neurohypophysial hormones (Fig. 2) and the blockade of the antagonodal activity of AVT by a vasopressor (but not by an oxytocic) antagonist, suggest stringent stereospecific requirements of the gonadal recognition site.

We and others have recently demonstrated that hypothalamic GnRH, in addition to its gonadotropin-releasing action in the pituitary, directly inhibits a variety of ovarian and testicular functions<sup>26–30</sup>. Our present findings indicate that central nervous system peptides other than GnRH can also directly inhibit testicular androgen biosynthesis. Use of the present bioassay system to screen synthetic analogues of neurohypophysial hormones for antagonodal activity might lead to the development of a new group of highly potent and selective contraceptive peptides capable of directly interfering with Leydig cell androgen biosynthesis.

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**Fig. 2** Correlation of the antagonodal potency of neurohypophysial hormones with their known antidiuretic (●), vasopressor (▲) and oxytocic (○) potencies. The relative antagonodal potency of neurohypophysial hormones is based on ED<sub>50</sub> values derived from Fig. 1. The relative antidiuretic, vasopressor and oxytocic potencies of neurohypophysial hormones in the rat have been published elsewhere<sup>18</sup>. AVP is used as the reference compound for antidiuretic, vasopressor and antagonodal activities whereas oxytocin is the reference compound for oxytocic activity. Reference compounds have been assigned the arbitrary potency value of 100. Antagonodal versus vasopressor activity,  $r = 0.97$ ; antagonodal versus antidiuretic activity,  $r = 0.96$ .

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## Amyloid P component is located on elastic fibre microfibrils in normal human tissue

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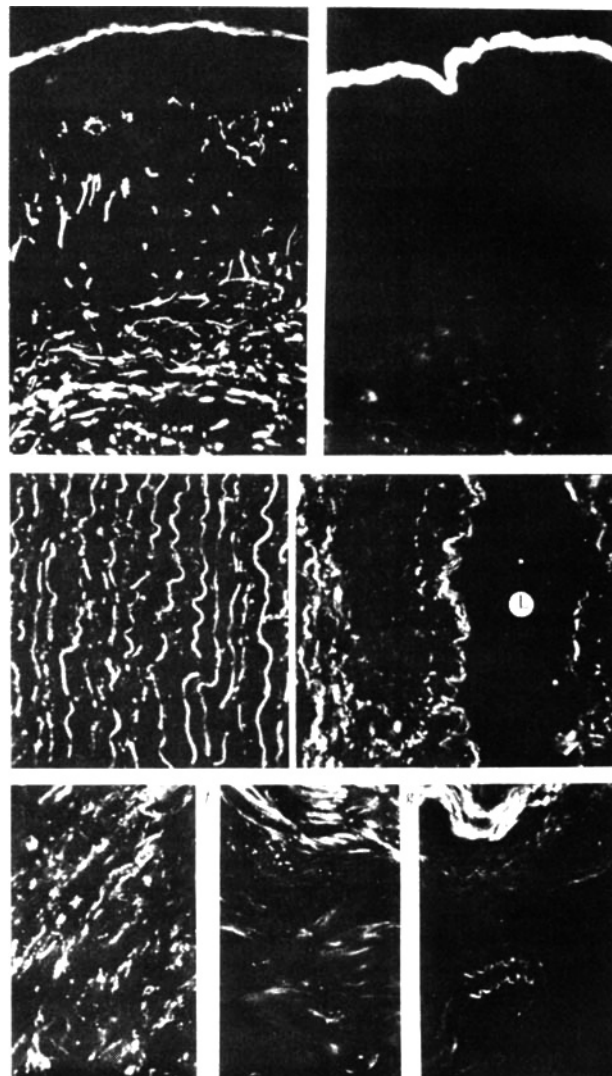
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Amyloid P component (AP) is a glycoprotein found in amyloid deposits<sup>1-3</sup> which is apparently identical to the normal plasma protein, serum amyloid P component (SAP)<sup>4</sup>. SAP closely resembles C-reactive protein (CRP), the classical acute phase reactant, in molecular configuration<sup>5,6</sup> and amino acid sequence<sup>7</sup> but is not related to any other known protein. We have recently shown that a protein which is immunohistochemically cross-reactive with SAP is an integral constituent of normal human glomerular basement membrane and is covalently associated there with collagen and/or other matrix proteins<sup>8-10</sup>. We now report that antibodies to SAP bind immunospecifically to the peripheral, microfibrillar mantle of elastic fibres in the skin and in blood vessels of normal adults. These findings extend both biochemical characterization of elastic fibres, which are a universal and important constituent of connective tissues, and knowledge of the normal tissue distribution of AP.

Microfibrils of average diameter ~8 nm are seen by transmission electron microscopy in all connective tissues<sup>11-13</sup>. They are most conspicuous as a peripheral mantle layer surrounding elastic fibres and are also frequently observed as a constituent of basement membranes bounding tissues and at endothelial surfaces. Apparently similar microfibrils may sometimes be found in association with, but distinct from, collagen fibres. Biochemical and immunochemical studies of microfibrils have been limited previously by the technical difficulties involved in their separation from other connective tissue elements and isolation in pure form<sup>13</sup>.

Here, direct immunofluorescence staining of unfixed normal human tissues with rabbit anti-human SAP antibodies produced bright fluorescence associated with elastic fibres in the connective tissue of the skin, lung, gut and heart, and in the walls of

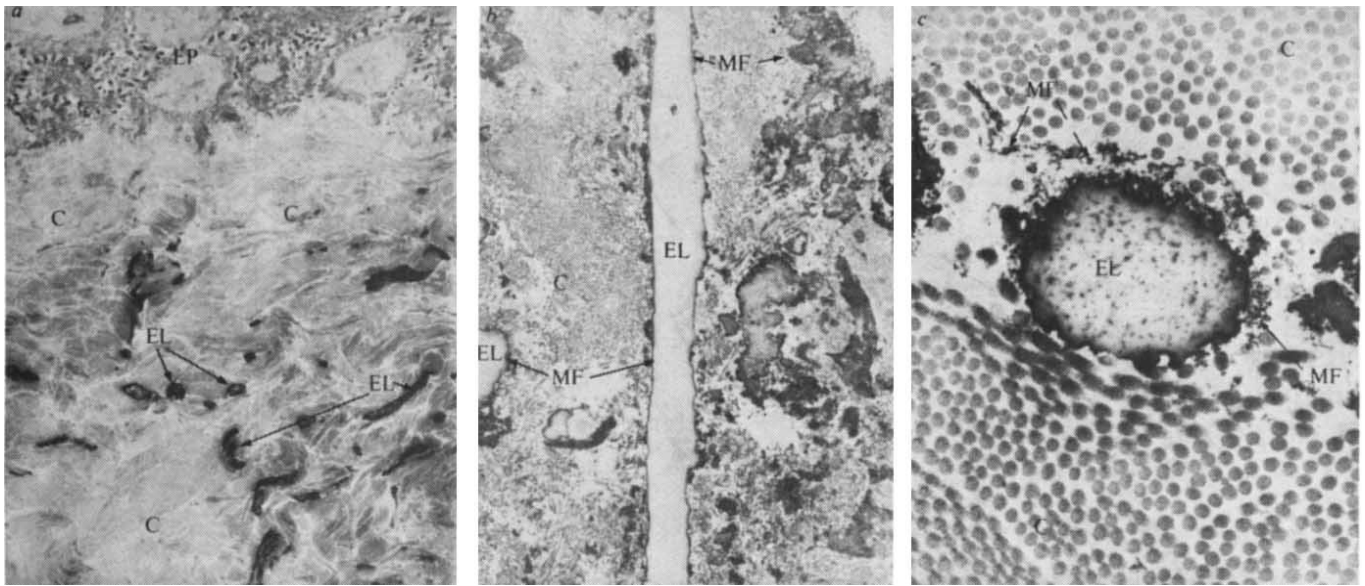


**Fig. 1** Immunofluorescence staining of normal human tissues with anti-SAP antibodies. Unfixed 4-µm cryostat sections of snap-frozen tissues obtained by biopsy or surgery were air-dried and washed with phosphate-buffered saline (PBS), pH 7.4. These were stained with fluorescein isothiocyanate-conjugated pepsin (Fab')<sub>2</sub> fragments of rabbit IgG anti-human SAP antibodies at an optimal dilution in PBS containing 1% w/v bovine serum albumin<sup>9,10</sup>. The antiserum, raised by immunization with isolated pure human SAP, was monospecific against whole human serum<sup>6</sup> in gel immunodiffusion tests. It did not stain any of four separate lines of cultured human skin fibroblasts. Control sections were stained with fluorescent antibody which had been preabsorbed with isolated pure human SAP<sup>6</sup>. Fluorescence was read using a Leitz Orthoplan microscope equipped with water immersion objectives and photographed with the Orthomat system using Kodak ASA 400 film. *a*, Skin; *b*, skin (control stained with preabsorbed fluor); *c*, media of the aorta; *d*, saphenous vein (L, lumen); *e*, myocardium; *f*, oesophageal wall; *g*, lung, ×110.

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**Fig. 2** Immunoperoxidase staining of normal human tissues with anti-SAP antibody. Blocks of fresh tissue were fixed in freshly prepared 3% w/v paraformaldehyde in 8.5% w/v sucrose in PBS for 10 min at 4 °C, washed thoroughly in sucrose/PBS and snap-frozen for cutting of 15- $\mu$ m cryostat sections. These were incubated with an optimal dilution of sheep IgG anti-human SAP antibodies which had been conjugated with horseradish peroxidase<sup>24</sup>. The antiserum, raised by immunization with isolated pure human SAP<sup>6</sup>, was monospecific against normal human serum in gel immunodiffusion tests and did not stain any of four separate lines of cultured human skin fibroblasts. The IgG fraction was obtained by DEAE-cellulose chromatography. Control sections were stained with peroxidase-conjugated anti-SAP which had been preabsorbed with isolated pure human SAP. After washing the tissue sections, bound conjugate was detected by incubation with diaminobenzidine and hydrogen peroxide<sup>25</sup>, post-fixing with osmium tetroxide and then embedding and resectioning for viewing in an AEI-801 transmission electron microscope. The electron-dense osmicated peroxidase reaction product is seen as a continuous layer on the surface of elastic fibres and the associated microfibrils. *a*, Skin (EP, epidermis; C, bundles of collagen fibres cut transversely and longitudinally; EL, elastic fibres).  $\times 1,590$ . *b*, Media of the aorta (C, collagen fibres; EL, elastin cores of elastic fibres sectioned longitudinally and transversely; MF, peripheral microfibrillar mantle of elastic fibres).  $\times 4,032$ . *c*, Skin (C, collagen fibres; EL, elastin core of elastic fibres containing intrinsic electron-dense fibrils; MF, same as for *b*).  $\times 15,750$ .

blood vessels (Fig. 1). In addition there was staining surrounding sweat glands in the skin, as described elsewhere<sup>14</sup>, and in some vascular basement membranes but not those of the dermal papillary vessels. Apart from the stratum corneum of the epidermis, which absorbed the fluor non-specifically, all fluorescent staining was completely inhibited by preabsorption of the anti-SAP reagent with isolated pure human SAP. The thin, superficial oxytalan fibres in the papillary dermis appeared to be stained homogeneously while the thicker elaunin and elastic fibres in the reticular dermis appeared as tubular structures stained only on the periphery. These results were confirmed by electron microscopy of sections of skin and aorta which had been stained with peroxidase-conjugated anti-SAP antibody. Specific binding of this reagent, which was completely inhibited when preabsorbed with pure SAP, was confined to the microfibrils making up the oxytalan and elaunin fibres and which are located at the periphery of mature elastic fibres (Fig. 2). There was no staining of collagen fibres or of the dermo-epidermal basement membrane.

SAP is not related to the microfibril antigens detected by an antiserum raised by Kewley *et al.*<sup>15,16</sup> against bovine elastic fibre microfibrils and used<sup>17</sup> to isolate microfibril-specific glycoproteins. Kewley's antiserum reacted with all morphologically recognizable microfibrils<sup>15,16</sup>, including those associated with collagen and with basement membranes generally. It also cross-reacted with human and avian microfibrils<sup>15,16</sup>, but not with human SAP (unpublished observations). In contrast, antisera to human SAP bind to glomerular but not renal tubular basement membrane<sup>8-10</sup>; they neither bind to collagen fibres in any tissue studied nor cross-react with bovine or avian SAP. AP itself contains no hydroxylated amino acids<sup>4</sup> and is not degraded by collagenase<sup>9</sup>, but it is released into solution, mainly in a high-molecular-weight form, by digestion of isolated glomerular basement membrane with collagenase<sup>9</sup>. Microfibrils are present on the endothelial aspect of the glomerular basement membrane<sup>18</sup> in the same position as the AP, as located by immuno-electron microscopy. Thus it is possible that, both there

and on elastic fibre microfibrils, AP may be associated with the microfibrillar protein, designated MFP I (ref. 17), which contains a collagenase-sensitive region. Further biochemical characterization of the AP and associated glycoproteins both in basement membrane and on elastic fibre microfibrils is in progress.

The physiological significance of AP as a normal tissue protein is unknown but it may be relevant that fibronectin, the only other protein that exists normally both in the plasma and in connective tissues<sup>19</sup>, is selectively bound *in vitro* by aggregated SAP<sup>20</sup>. With regard to pathophysiological processes, particularly amyloidogenesis, it is intriguing that there is often a close morphological association *in vivo* between amyloid fibrils and microfibrils of both elastic fibres<sup>21</sup> and basement membranes<sup>22</sup>, while SAP is known to bind specifically to isolated amyloid fibrils *in vitro*<sup>23</sup>.

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## Neurotoxic action of methyltetrahydrofolate in rat cerebellum unrelated to direct activation of kainate receptors

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Kainic acid is a potent neuroexcitant with neurotoxic properties—its infusion into the cerebellar cortex of rat and hamster selectively destroys inhibitory neurones<sup>1–3</sup>. Electrophysiological<sup>4</sup> and neurochemical<sup>5</sup> data indicate that kainate is unlikely to act through the predominant type of receptor for L-glutamate. Thus, kainate may interact either with a specific subclass of glutamate receptor or with receptors for an unidentified endogenous neuroexcitant. Recently, it was reported that the pteroylmonoglutamate compound, methyltetrahydrofolate (MTHF), is a potent inhibitor of <sup>3</sup>H-kainate binding to rat cerebellar membranes, while not influencing the binding of L-glutamate, and exhibits an activity approximately 1/10th of that of kainate itself<sup>6</sup>. As MTHF, like kainate, has convulsive properties<sup>7,8</sup>, we have investigated the possibility that this substance also exhibits neurotoxic effects. Here, we report that 2 weeks after intracerebellar injections of MTHF (250 nmol) there was a substantial loss of Purkinje cells and a reduction in associated markers for functions mediated by  $\gamma$ -aminobutyric acid (GABA). However, the time course of degeneration after MTHF was much slower than with kainate, and in contrast to kainate and other neurotoxic amino acids, MTHF did not increase the levels of cyclic GMP in cerebellar slices. A direct effect of MTHF on receptors for kainate therefore seems unlikely.

As reported previously<sup>3</sup>, injection of kainate (10 nmol) at three sites in the rat cerebellum resulted within 48 h in an extensive loss of Purkinje and other inhibitory cell types as compared with control tissue (Fig. 1a, b). In preliminary experiments, MTHF was injected at a dose (triple injections of 100 nmol) directly comparable with that of kainate on the basis of the <sup>3</sup>H-kainate binding data<sup>6</sup>. However, this could serve only as an approximation because MTHF is rapidly metabolized (and hence possibly inactivated) by the enzyme MTHF-homocysteine methyltransferase.

After 48 h, in contrast to the effects of kainate, no cell loss was observed. However, a variable proportion (10–20%) of the Purkinje cells appeared significantly shrunken and pyknotic (data not shown). Histological and biochemical assessment of cerebella injected with a higher dose of MTHF (triple injections of 250 nmol) administered 14 days previously, indicated extensive neurotoxicity (Fig. 1c), with an approximate 40% loss of Purkinje cells and a further 30% of these cells appearing pyknotic, as estimated from serial 10- $\mu$ m parasagittal sections taken either side of the injection sites, and comprising about one-third of the cerebellum. In contrast to the Purkinje cells, granule cells appeared normal. Compared with control tissues,

**Table 1** Effects of MTHF and threo-3-hydroxyaspartate on cerebellar GAD activity and GABA uptake

| Treatment                | GAD activity (mmol GABA formed per kg per h) | P2 GABA uptake (pmol per mg protein per 3 min) |
|--------------------------|----------------------------------------------|------------------------------------------------|
| Control                  | 30.5 $\pm$ 0.43                              | 177.1 $\pm$ 27.2                               |
| MTHF                     | 24.6 $\pm$ 1.00*                             | 140.9 $\pm$ 17.8                               |
| Threo-3-hydroxyaspartate | 31.8 $\pm$ 0.91                              | 158.8 $\pm$ 11.3                               |

Rats were injected with MTHF or threo-3-hydroxyaspartate as described in Fig. 1 legend. After 14 days cerebella were removed following decapitation of the animals, and the tissues were homogenized in 20 vol 0.32 M sucrose. Glutamate decarboxylase activity and high-affinity <sup>3</sup>H-GABA uptake were determined precisely as described previously<sup>3</sup>. Results are means  $\pm$  s.e.m. of triplicate determinations with three animals for each treatment.

\*Statistical significance of difference from control by *t*-test, *P* < 0.001.

glutamate decarboxylase (GAD) activity of MTHF-injected cerebella was reduced (by ~20%), as was GABA uptake into P2 preparations, although the reduction in the latter was not statistically significant (Table 1).

Glutamate and kainate markedly increased cyclic GMP levels in 8-day old rat cerebellar slices<sup>9</sup> whereas MTHF at concentrations of up to 1 mM failed to increase cyclic GMP levels (Table 2). Additionally, whereas kainate and glutamate exhibited a marked synergistic effect on the stimulation of cyclic GMP levels, no such interaction occurred with MTHF. Other biochemical studies involving excitatory amino acid-induced ion fluxes in adult rat striatal slices have shown that kainate and other neurotoxic amino acid analogues produce large increases in <sup>22</sup>Na<sup>+</sup> flux rates, while MTHF is inactive (ref. 10 and V. I. Teichberg, personal communication). This suggests that MTHF does not act in the same way as kainate.

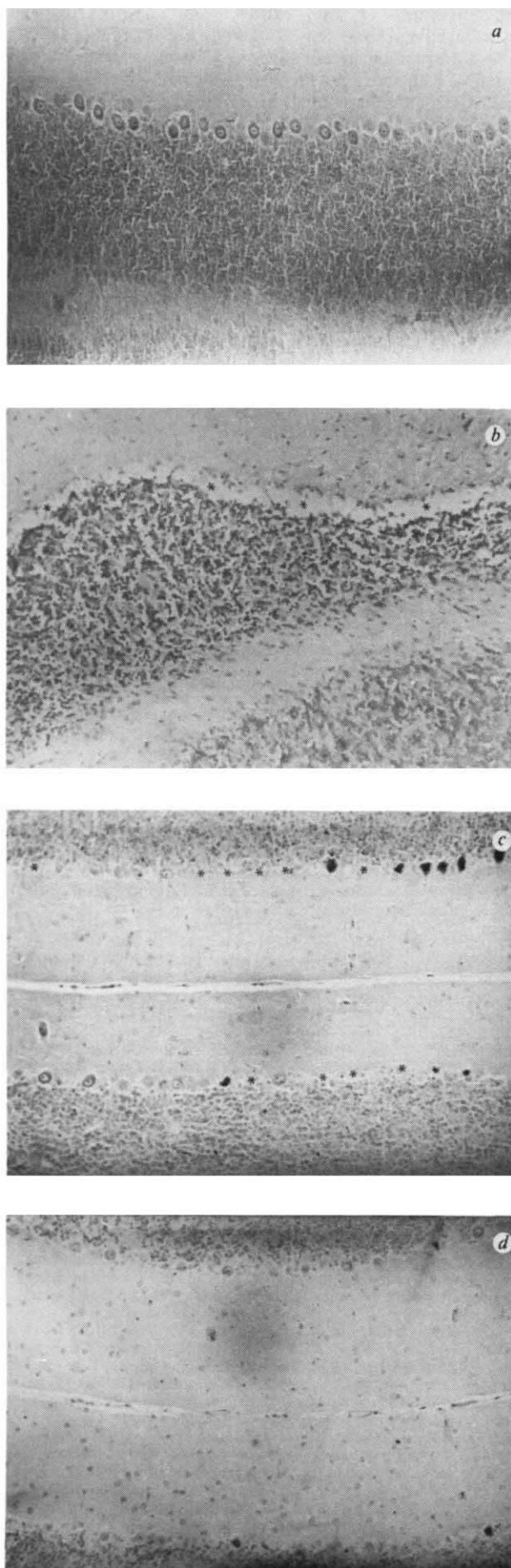
Folate and two of its analogues (*N*<sup>5</sup>-formyl-5,6,7,8-tetrahydrofolate and 4-amino-*N*<sup>10</sup>-methylpteroylglutamic acid) have powerful facilitatory actions on glutamate-activated or spontaneously firing cat pericruciate cortex neurones, but possess only weak excitatory actions on quiescent neurones<sup>11</sup>. This effect may in part be due to the ability of these agents to inhibit high-affinity glutamate uptake<sup>12</sup>. We have accordingly examined the ability of MTHF to inhibit synaptosomal uptake of <sup>3</sup>H-D-aspartate (a non-metabolizable substrate for the glutamate uptake carrier), and have found it to be approximately 1/100th as effective as the potent glutamate uptake inhibitor,

**Table 2** Effect of L-glutamate, kainate and MTHF on cerebellar cyclic GMP levels

| Compound (1 mM)                | Cyclic GMP formed (pmol per mg protein) | % Stimulation |
|--------------------------------|-----------------------------------------|---------------|
| Control                        | 0.3 $\pm$ 0.1                           |               |
| L-glutamate                    | 29.9 $\pm$ 3.6                          | 6,000         |
| Kainate                        | 19.1 $\pm$ 1.7                          | 3,820         |
| MTHF                           | 0.4 $\pm$ 0.2                           | —             |
| L-glutamate + kainate (0.1 mM) | 105.9 $\pm$ 2.7                         | 21,180        |
| L-glutamate + MTHF             | 33.3 $\pm$ 2.7                          | 6,660         |

Cerebellar slices (0.5  $\times$  0.5 mm) from 8-day-old rats were prepared on a McIlwain chopper and preincubated in Krebs-bicarbonate medium at 37 °C under 95% O<sub>2</sub>/5% CO<sub>2</sub>. After 90 min, the compounds under test were added and the incubation was continued for a further 5 min. Tissue concentrations of cyclic GMP were determined by radioimmunoassay as described previously<sup>9</sup>. Results are means  $\pm$  s.e.m. of at least four independent observations. In the experiments with combinations of compounds, the response to L-glutamate + kainate was significantly different (*P* < 0.001) from the sum of the effects of kainate and glutamate alone.





**Fig. 1** Effects of intracerebellar injections of kainate, MTHF and threo-3-hydroxyaspartate. Female Wistar rats (four per group) weighing 200–250 g were anaesthetized with sodium pentobarbitone (60 mg per kg intraperitoneally), placed in a stereotaxic frame, and triple injections of kainate (10 nmol), MTHF (250 nmol) or threo-3-hydroxyaspartate (170 nmol) in phosphate-buffered saline (pH 7.4) were made 5 mm back from lambda at sites on and 2.5 mm either side of the midline, to a depth of 3 mm. Because of the relative instability of MTHF in solution, this was prepared immediately before injection and kept at +2 °C, protected from light. Animals were allowed to survive for 48 h (kainate-treated) or 14 days before being anaesthetized and transcardially perfused-fixed with Heidenhain's Susa. Cerebella were embedded in wax and 10- $\mu$ m sections stained with cresyl violet. *a*, Control cerebellum after injection 14 days previously with vehicle. *b*, Kainate (10 nmol) injected cerebellum; note complete loss of Purkinje cell layer (asterisks). *c*, MTHF (250 nmol) injected cerebellum. Extensive loss of Purkinje cells (asterisks), numerous shrunken and pyknotic cells. *d*, Threo-3-hydroxyaspartate (170 nmol) injected. Shrunken Purkinje cells apparent: no cell loss observed.  $\times 14$ .

threo-3-hydroxyaspartate. Using a cerebellar P2 preparation, the uptake of  $^3\text{H}$ -D-aspartate was examined over a wide range of inhibitor concentrations and log dose-inhibition curves constructed (not shown). These yielded  $\text{IC}_{50}$  values of  $2.5 \times 10^{-6}$  and  $4.0 \times 10^{-4}$  M for threo-3-hydroxyaspartate and MTHF, respectively.

Injection of a high dose of threo-3-hydroxyaspartate (triple injections of 170 nmol) into the cerebellum resulted in some apparent neurotoxicity when assessed 2 weeks later. However, this was very much less than that occurring with MTHF (Fig. 1*d*) and no significant alteration in either GABA uptake or GAD activity was observed (Table 1).

In conclusion, we have found that following intracerebellar injections, MTHF at relatively high doses exerts a marked neurotoxicity similar to that seen following injection of kainate or other 'excitotoxic' amino acids. However, we do not know whether the MTHF lesion is axon-sparing. Although binding data from one research group indicate that MTHF may interact potently with kainate receptors<sup>6</sup>, other biochemical assays have failed to reveal a kainate-like action, and this, coupled with the much slower time course of neurotoxicity observed with MTHF, may suggest an alternative mechanism. It is feasible, for example, that MTHF or a metabolite might exert its toxicity at least in part by interfering with the uptake of glutamate or other similar endogenous excitant. In this respect it is pertinent that destruction of glutamate uptake sites produces a dramatic enhancement of glutamate toxicity in the hippocampus<sup>13</sup>.

Following submission of our findings, Olney *et al.* reported<sup>14</sup> that MTHF (300 nmol) injected into the rat amygdala and folic acid (50 or 100 nmol) in the striatum failed to mimic the local neurotoxic actions of kainate, while duplicating the kainate pattern of distant brain damage which is probably attributable to a seizure mechanism. These findings therefore also indicate that major differences exist between the actions of kainate and MTHF in effecting neurotoxicity.

G.A.F. is an SRC research student.

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## New vertebrate brain-gut peptide related to a molluscan neuropeptide and an opioid peptide

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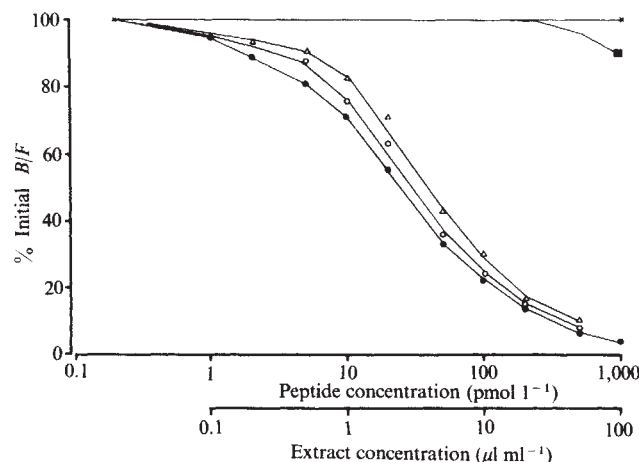
Peptides related to mammalian hormones and neuropeptides are known to occur widely in invertebrate nervous systems<sup>1-11</sup>. It seems plausible to suppose that peptides which have hitherto been found only in invertebrate nervous systems might also have representatives that function as hormones or neuroregulators in vertebrates. However, this idea has yet to be rigorously tested. Recently, Boer *et al.*<sup>12</sup> described immunohistochemical studies in which antisera to a peptide isolated from molluscan ganglia, Phe-Met-Arg-Phe-NH<sub>2</sub> (FMRFamide)<sup>13</sup>, revealed nerve fibres in the brain of the mouse and a teleost fish. This tetrapeptide is of particular interest in that its amino acid sequence is related to that of the C-terminal tetrapeptide of cholecystokinin (CCK) and gastrin (Trp-Met-Asp-Phe-NH<sub>2</sub>), and is identical to the primary sequence of the C-terminal tetrapeptide of an enkephalin-related heptapeptide, Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH, recently isolated from bovine adrenal medulla and striatum and known to be a potent opioid agonist<sup>14-16</sup>. It is not known whether the material identified by Boer *et al.* corresponds to these previously identified vertebrate peptides. We present here evidence from radioimmunoassay and immunohistochemical studies that FMRFamide-like material occurs in central nervous system and gut endocrine cells of vertebrates. The material is distinguishable in properties and distribution from presently known peptides related to CCK or Met-enkephalin, and we propose that it be added to the list of substances thought to have dual roles as gut hormones and central neuroregulators<sup>17,18</sup>.

Antisera to FMRFamide were obtained by immunization of rabbits with synthetic FMRFamide coupled to thyroglobulin by glutaraldehyde. In radioimmunoassays using Tyr<sup>1</sup>-FMRFamide labelled with <sup>125</sup>I, the concentration of FMRFamide needed for 50% inhibition of binding of label was 20–25 fmol ml<sup>-1</sup> (Fig. 1). Cleavage of FMRFamide between arginine and phenylalanine by incubation with trypsin, reduced immunochemical potency over 1,000-fold. The antiserum failed to show significant affinity (<0.001 relative to FMRFamide) for the following peptides: C-terminal octa- and tetrapeptides of cholecystokinin, vasoactive intestinal polypeptide, substance P, bombesin, C-terminal hexapeptide of pancreatic polypeptide,  $\beta$ -endorphin, Met-enkephalin, Met-enkephalin-[Arg<sup>6</sup>], Met-enkephalin-[Lys<sup>6</sup>], Met-enkephalin-NH<sub>2</sub>, Leu-enkephalin-[Arg<sup>6</sup>], Leu-enkephalin-[Lys<sup>6</sup>]. Moreover, the desamido tetrapeptide (FMRF-OH) had 100-fold lower immunoreactivity than the amidated form (Fig. 1). Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>], that is, the desamido tetrapeptide extended at the N-terminus by Tyr-Gly-Gly, also failed to show significant immunochemical potency (Fig. 1). In sharp contrast, the pentapeptide produced when FMRFamide was extended at the N-terminus by tyrosine showed full immunochemical potency. These results therefore demonstrate that the antiserum has high specificity for the C-terminus of FMRFamide, and in particular requires the C-terminal amide for full immunoreactivity.

Initial studies on extracts of brain, gastrointestinal tract and pancreas of several vertebrate species (rat, cow, dog, frog and chicken) indicated that relatively high concentrations of immunoreactive material occurred in frog brain (163  $\pm$  27 pmol per g, mean  $\pm$  s.e.,  $n$  = 5) and chicken pancreas (93  $\pm$  9 pmol per g,  $n$  = 5), and these tissues were therefore selected for detailed studies. Extracts of both tissues inhibited binding of label to antiserum in parallel with standard FMRFamide (Fig. 1). In both

cases, digestion of the extract with trypsin reduced immunoreactivity by at least 100-fold (Fig. 1). These results establish the peptidic nature of the vertebrate material, and are compatible with an arginine residue in the penultimate position, as in FMRFamide. Fractionation of both extracts on Sephadex G-50 revealed a major peak of activity that eluted significantly before FMRFamide (Fig. 2). Sephadex gel filtration is an imperfect guide to molecular weight of small peptides, but it is likely that the FMRFamide-like immunoreactive factors in frog brain and chicken pancreas are larger than the molluscan tetrapeptide, and presumably correspond to extensions at the N-terminus of the antigenic determinant.

The cellular distribution of FMRFamide immunoreactivity was studied by an indirect immunofluorescence method<sup>19</sup>. Frog

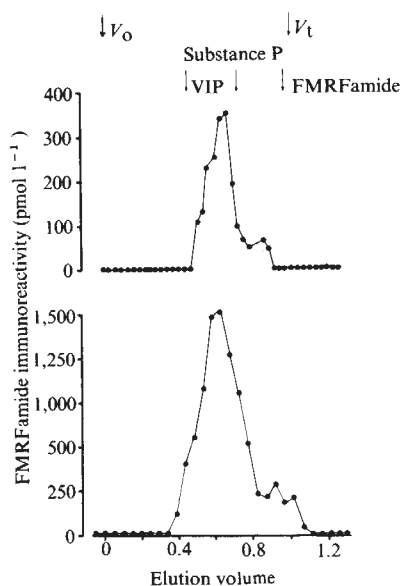


**Fig. 1** Inhibition of binding of <sup>125</sup>I-labelled [Tyr<sup>1</sup>]-FMRFamide to antiserum by graded concentrations of standard peptides or tissue extracts. Antiserum was used at a dilution of 1:25,000, and incubated in phosphate buffer (0.05 M, pH 7.4, containing 0.14 M sodium chloride, 3  $\mu$ M sodium azide and 0.5% v/v Bovumim) with 2,000 c.p.m. of label. The antiserum was raised in a rabbit immunized with a conjugate prepared by reacting (30 min, 22 °C) FMRFamide (500 nmol), thyroglobulin (2 mg) and glutaraldehyde (20  $\mu$ l, 5% solution added to 1.0 ml of the other reactants in 0.05 M phosphate buffer, pH 7.4), followed by dialysis against distilled water (4.14 °C, 24 h). The rabbit was immunized with the equivalent of 75 nmol FMRFamide, by a schedule previously used<sup>21</sup> for CCK8. [Tyr<sup>1</sup>]-FMRFamide (Peninsula) was iodinated by the chloramide-T method and purified on CM-Sephadex eluted with ammonium acetate. Separation of antibody-bound (B) and free (F) label in the radioimmunoassay was obtained by addition of 100  $\mu$ l of charcoal suspension (100 g l<sup>-1</sup>) precoated with dextran (10 g l<sup>-1</sup>) and plasma (100 ml l<sup>-1</sup>), and centrifugation (2,000g, 10 min). Inhibition of binding by standards or extracts is expressed as percentage of the initial ratio of bound to free counts in the absence of competing peptide. Tissue extracts were prepared by boiling tissues in water (0.1 g ml<sup>-1</sup>; 5 min) and centrifuging (2,000g, 10 min), the residues were re-extracted with 0.5 M acetic acid, centrifuged as before and the two extracts pooled. Re-extraction of pellets revealed >90% of activity recovered in the first extraction cycle. The same method was used to prepare extracts of brain from rat and cow, and of alimentary tract of rat, dog and chicken. Partial purification of some extracts was achieved by adsorption to Amberlite XAD<sub>2</sub> resin, elution with 90% methanol and lyophilization; recovery of FMRFamide by this method was >90%. Trypsinized extracts were prepared by incubation (37 °C, 1 or 18 h) of extracts in ammonium bicarbonate (0.05 M) together with 50  $\mu$ g ml<sup>-1</sup> of TPCK-trypsin (Worthington), followed by inactivation in a boiling water bath (5 min). Specificity of the assay was established by studies of cross-reactivity of the following peptides: FMRFamide (Peninsula), pure natural porcine vasoactive intestinal polypeptide (VIP; donated by Professor V. Mutt), synthetic substance P (Peninsula), synthetic sulphated C-terminal octapeptide of cholecystokinin (CCK8; Squibb), synthetic C-terminal tetrapeptide of gastrin/cholecystokinin (G4; donated by Professor G. W. Kenner), FMRF-OH (donated by Dr I. Galpin), synthetic tetradecapeptide bombesin (donated by Farmitalia), Met-enkephalin and Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>] (Peninsula). The following peptides were also studied and found to have no significant immunochemical potency (<0.001 relative to FMRFamide): C-terminal hexapeptide of pancreatic polypeptide (donated by Dr I. Taylor),  $\beta$ -endorphin (donated by Dr R. Guilleman), Met-enkephalin-[Arg<sup>6</sup>], Met-enkephalin-[Lys<sup>6</sup>], Met-enkephalin-NH<sub>2</sub>, Leu-enkephalin-[Arg<sup>6</sup>] and Leu-enkephalin-[Lys<sup>6</sup>] (all Peninsula). ●, FMRFamide; ○, chicken pancreas; △, frog brain; ■, FMRF-OH; ×, CCK8, G4, Met-enkephalin, Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>], substance P, bombesin, frog brain (trypsin) and chicken pancreas (trypsin).

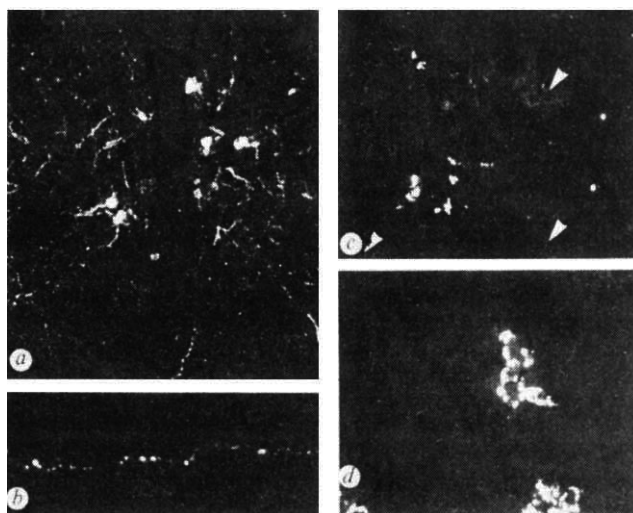


brain showed an extensive system of immunofluorescent nerve cell bodies and nerve fibres around the ventricle in the preoptic region (Fig. 3). Moreover, numerous immunoreactive fibres were also identified around the lateral ventricles in the fore-brain, in the thalamus, hypothalamus, optic tectum and medulla but were scarce in the cerebellum. Studies on rat brain have revealed immunofluorescent fibres particularly in the nucleus accumbens, medial thalamus, hypothalamus, brain stem and spinal cord (Fig. 3). However, in addition to a neuronal origin, immunohistochemical studies have demonstrated abundant immunoreactive endocrine-like cells in gastrointestinal tract and pancreas. In the chicken, numerous fluorescent cells were found scattered throughout the acinar tissue of the pancreas, and were not localized in islets (Fig. 3). In the gastrointestinal tract immunofluorescent endocrine-like cells were common in the ileum and colon mucosa, particularly in the dog. In brain, gut and pancreas, the distribution of cells with FMRFamide-like immunoreactivity was readily distinguishable from that of cells demonstrated by antisera to Met-enkephalin or CCK. Moreover, antisera to FMRFamide failed to demonstrate immunoreactive cells in the dog or bovine adrenal medulla, which are known to contain enkephalin-like peptides<sup>14</sup>, and which were readily stained by Met-enkephalin antisera. The positive FMRFamide-like immunoreactivity in brain and gut was abolished by preabsorption of antiserum with FMRFamide (10 nmol ml<sup>-1</sup>), but not by absorption with Met-enkephalin, Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>] or CCK8 (all 100 nmol ml<sup>-1</sup>), indicating the specificity of localization.

Full characterization of the material identified here must await its isolation, and work in this direction is in progress. Conceivably the FMRFamide-like activity could be due to C-terminally amidated forms of Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>], or an N-terminally extended variant<sup>20</sup>. It is noteworthy, therefore, that in bovine adrenal medulla and rat and bovine striatum<sup>14,16</sup>, tissues that contain Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>] in significant quantities (up to 50 pmol per g), we have found only trace amounts of FMRFamide-like immunoreactivity (<5 pmol per g). Thus, for a common precursor to give rise to Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>] on the one hand and FMRFamide-like



**Fig. 2** Fractionation on Sephadex G-50 superfine (1 × 100 cm) of extracts of chicken pancreas and frog brain (see Fig. 1). Columns were run in 0.5 M acetic acid at 4 °C. As acetic acid caused nonspecific inhibition in the assay, all eluate tubes were lyophilized and reconstituted in assay buffer before assay. Top arrows indicate elution positions of substances used to calibrate the column; bovine serum albumin ( $V_0$ ), pure natural porcine VIP, synthetic substance P, synthetic FMRFamide and sodium chloride ( $V_1$ ). Recovery of immunoactivity from columns was 80–100%.



**Fig. 3** Tissues for immunohistochemistry were taken from brain (frog, rat), gastrointestinal tract and pancreas (frog, chicken, rat, cat, dog, pig). The tissues were fixed in 0.4% *p*-benzoquinone in 0.1 M sodium cacodylate buffer, pH 7.3, for 1–4 h at 4 °C, rinsed for 24 h in the same buffer containing 20% sucrose, then snap-frozen and sectioned in a cryostat. 10-μm sections were incubated for 24 h with antiserum to FMRFamide (at dilutions up to 1:400), then incubated for a further hour with goat-anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:40, Miles). Following both incubations, sections were rinsed for 1 h in phosphate-buffered saline. FMRFamide-like immunoreactivity localized in: *a*, nerve cell bodies and fibres around the third ventricle in the preoptic region of frog brain, section counterstained with haematoxylin, ×85; *b*, a beaded nerve fibre in rat thalamus, ×440; *c*, cells scattered in the chicken pancreas outside islets (arrows)—the latter are clearly demonstrated in a serial section (*d*) stained with an antiserum to somatostatin (7812, 1:500, a gift from Dr T. Yamada), ×60. Non-immune serum gave negative results; for further absorption controls see text.

peptides on the other, would require separate patterns of post-translational processing in the different cell types. This possibility requires serious consideration because recent findings in our laboratory indicate that synthetic FMRFamide has potent excitatory actions on brain stem neurones in the rat which are clearly distinct from the mainly inhibitory actions of Met-enkephalin or Met-enkephalin-[Arg<sup>6</sup>-Phe<sup>7</sup>] (R. J. Gayton, personal communication). However, regardless of the biosynthetic origins of the FMRFamide-like material, the present studies establish these peptides as candidates for neuroregulatory and endocrine functions in vertebrates, and so provide evidence that invertebrate neuropeptides have vertebrate counterparts.

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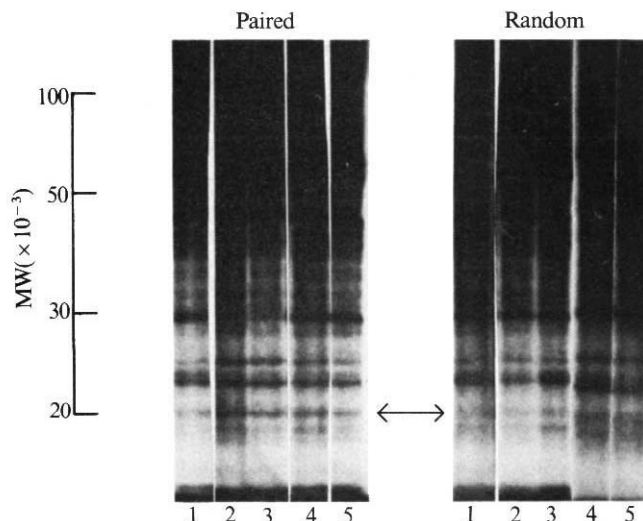
## Change in a specific phosphoprotein band following associative learning in *Hermisenda*

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Although cellular neurophysiological studies of invertebrates have provided some understanding of the cellular basis of modifiable behaviour (for reviews, see refs 1–5), little is known about biochemical changes in the specific neural pathways accompanying alterations in behaviour. The nervous systems and sensory structures of invertebrates are especially suitable to an analysis of the biochemical mechanisms involved in behavioural modification because they typically contain a relatively small number of neurones, some of which have large, identifiable somata, and because associative learning has been studied in several invertebrate preparations<sup>6–10</sup>. Studies with the nudibranch mollusc *Hermisenda crassicornis* have demonstrated a short-term non-associative change in phototactic behaviour after training with light and rotation<sup>11</sup>. More recently<sup>8,12</sup>, an example of associative learning in *Hermisenda* was produced by the temporal association of light and a presumed aversive stimulus. This associative learning is accompanied by neurophysiological changes in the B-type photoreceptors<sup>12</sup>. We now report that there is also a change in the level of incorporation of <sup>32</sup>P in a 20,000-molecular weight (MW) phosphoprotein; as the eye of *Hermisenda* consists of only five photoreceptors, a lens and a few pigment and epithelial cells, a biochemical change specific to associative learning has thus been localized to a few cells within a nervous system.

*Hermisenda* were trained by the following conditioning procedure<sup>8</sup>. First, the times taken (latency) by individual animals to enter an illuminated area were measured to establish individual baselines, and the animals were then trained in an automated apparatus<sup>14</sup> by a protocol involving stimulation of the eyes and of the gravity-detecting statocysts by light and rotation respectively. Trained animals received simultaneous light and rotation (paired); control animals were presented with light and rotation on independent random schedules (random control) or unpaired light and rotation (explicitly unpaired). All groups received the same number of light and rotation trials. One trial consisted of the presentation of light and/or rotation for 30 s with an intertrial interval of 1–3 min. The animals received 50 trials per day for 3 days, after which the latency of each animal to enter an illuminated area was measured and compared with its pre-training value. Latencies were greater for animals trained with paired light and rotation than for those in random or unpaired control groups<sup>8</sup>. After testing, the circumoesophageal nervous system, consisting of eyes, statocysts and ganglia, was dissected from each animal and incubated separately in artificial seawater containing glucose and <sup>32</sup>P<sub>i</sub> for 2 h at 15 °C (see Fig. 1 legend). Incorporation of phosphate into protein is linear with respect to time for 1–4 h (ref. 15 and J.T.N., unpublished observations). After rinsing the nervous systems in an ice-cold isotonic solution, the eyes and a small amount of surrounding connective tissue were dissected on a cold plate and lysed. Aliquots of eye samples from individual animals were then analysed for (1) total phosphoproteins by trichloroacetic acid precipitation and (2) specific phosphoprotein bands by SDS-polyacrylamide gel electrophoresis<sup>16</sup> and autoradiography<sup>17</sup>.



**Fig. 1** SDS electrophoresis gels comparing endogenous protein phosphorylation in eyes of *Hermisenda* presented with paired or random light and rotation. *Hermisenda* from Monterey, California, were fed daily and maintained in 6.5-h light per day for 3 days before testing and training. After 3 days of training with paired or random light and rotation as previously described<sup>8</sup>, the circumoesophageal nervous system was dissected from each animal, transferred to a vial containing 200  $\mu$ l of artificial seawater (460 mM NaCl, 10 mM KCl, 50 mM MgCl<sub>2</sub>, 11 mM CaCl<sub>2</sub> and 50 mM Tris-HCl pH 7.6), and incubated at 15 °C for 30 min. Each nervous system was then transferred to a vial containing 200  $\mu$ l of oxygenated artificial seawater, 11 mM glucose and 0.125 mCi <sup>32</sup>P<sub>i</sub> (carrier free, NEN), incubated in a covered rack for 2 h at 15 °C and quickly rinsed four times in 1-ml portions of an isotonic, ice-cold rinse solution (460 mM NaCl, 10 mM KCl, 5 mM EDTA and 100 mM Tris-HCl pH 7.6). Eyes were dissected on a cooling plate, lysed in a modified lysis solution<sup>37</sup> containing 9.3 M urea, 0.5% SDS, 5%  $\beta$ -mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride and 5 mM EDTA (10  $\mu$ l per eye), and stored frozen at -70 °C. Aliquots of eye samples from individual animals were analysed by SDS-polyacrylamide gel electrophoresis<sup>16</sup> in 0.75-mm slab gels (4.7% stacking gel, 11% running gel). Gels were stained and fixed in 0.1% Coomassie blue, 50% trichloroacetic acid for 30 min, destained overnight in 7% acetic acid and dried. Molecular weights were estimated by comparison with mobilities of standard proteins of known molecular weight (myosin,  $\beta$ -galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and cytochrome *c*). Autoradiography was conducted with Kodak X-Omat R film, flashed to 0.15 optical density and a Cronex Lightning Plus intensifying screen (Dupont)<sup>17</sup>. Exposure times were chosen to obtain a linear relationship between radioactivity and grain density for each band: overnight exposure (16–20 h) to visualize the major phosphoproteins, 2–3-day exposure for minor phosphoprotein bands. The protein nature of the radioactive bands was indicated by their susceptibility to degradation by pronase and by their resistance to ribonuclease A treatment and chloroform-methanol extraction (ref. 15 and J.T.N., unpublished observations). Each lane represents an eye sample from one animal. The autoradiograms shown here were exposed for 3 days. As indicated by Table 1, the extensively labelled phosphoprotein bands from eyes of animals receiving paired light and rotation were not significantly different from those presented with random stimuli (autoradiograms of overnight exposures not shown).

In the past year, four independent replicates of the paired paradigm (total  $n = 16$ ), three independent replicates of the random paradigm (total  $n = 12$ ) and one unpaired group ( $n = 5$ ) have been examined. Animals which received no training (untreated) but were maintained on the same light cycle, feeding schedule and temperature as experimental and control animals were studied in parallel. No significant differences were found in the overall level of phosphate incorporated into total protein in the eyes of animals in the four groups.

Phosphoprotein bands of eye samples from individual animals of all groups were visualized by autoradiography of SDS-electrophoresis gels. An increase was observed in the level of <sup>32</sup>P incorporation in a 20,000-MW phosphoprotein band in the eyes from animals presented with paired light and rotation (Figs 1, 2). An analysis of variance revealed an overall significant difference ( $F_{2,30} = 6.49$ ;  $P < 0.01$ ) among the groups in the 20,000-MW phosphoprotein band (Table 1). Planned comparisons (two-tailed  $t'$ -tests) revealed that the paired group

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was significantly different ( $P < 0.01$ ) from the random and unpaired control groups while the two controls were not different from each other. The amount of incorporation in the 20,000-MW phosphoprotein band from eyes of animals receiving paired stimuli was about twice that in eyes from untreated animals and 80% more than in eyes from animals receiving random or unpaired stimuli. The 20,000-MW phosphoprotein band constitutes ~1% of the total phosphoproteins in the eyes of untreated animals.

No significant differences were observed in 10 other phosphoprotein bands which were detectable in eyes from all groups studied (Table 1). In some cases ( $n = 5$ ), a second minor phosphoprotein band (25,000 MW) was observed in eyes from animals receiving paired stimuli; although this band has not been consistently present, we have never observed significant levels of it in eyes from random, unpaired, or untreated animals.

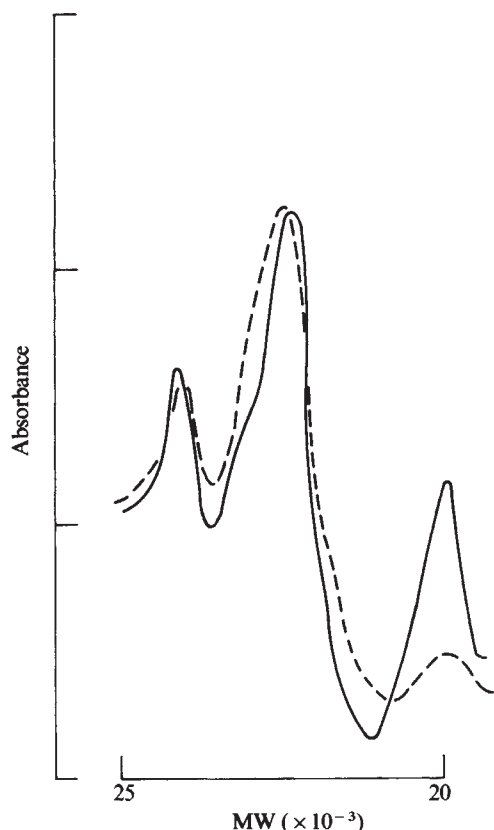
Compared with the eyes of vertebrates, the eye of *Hermisenda* is a relatively simple structure, containing five photoreceptors, a lens, pigment and epithelial cells<sup>18,19</sup>. Photoreceptors send axons through the optic ganglia into the cerebropleural ganglia. The synaptic interactions of the photoreceptors with themselves and with other neurones in the visual and statocyst pathways have been examined in detail<sup>20-22</sup>. In trained animals, the light responses of type-B photoreceptors include a light-evoked tonic depolarization, increases in the spontaneous spike frequency and increases in the input resistance<sup>12</sup>. A long-lasting depolarizing response of type-B photoreceptors following stimulation of isolated nervous systems by

**Table 1** Effect of paired, random and unpaired stimulation on specific phosphoprotein bands in *Hermisenda* eyes

| Phosphoprotein band (MW) | Ratio of experimental/untreated (mean $\pm$ s.e.m.) Paired ( $n = 16$ ) | Ratio of experimental/untreated (mean $\pm$ s.e.m.) Random ( $n = 12$ ) | Ratio of experimental/untreated (mean $\pm$ s.e.m.) Unpaired ( $n = 5$ ) |
|--------------------------|-------------------------------------------------------------------------|-------------------------------------------------------------------------|--------------------------------------------------------------------------|
| 72,000                   | 0.83 $\pm$ 0.08                                                         | 1.05 $\pm$ 0.23                                                         | 1.30 $\pm$ 0.38                                                          |
| 55,000                   | 1.02 $\pm$ 0.04                                                         | 1.04 $\pm$ 0.09                                                         | 0.88 $\pm$ 0.07                                                          |
| 44,000                   | 0.88 $\pm$ 0.12                                                         | 1.35 $\pm$ 0.10                                                         | 1.04 $\pm$ 0.44                                                          |
| 42,000                   | 1.27 $\pm$ 0.14                                                         | 1.18 $\pm$ 0.08                                                         | 1.08 $\pm$ 0.13                                                          |
| 38,000                   | 1.11 $\pm$ 0.07                                                         | 0.87 $\pm$ 0.10                                                         | 1.23 $\pm$ 0.14                                                          |
| 34,000                   | 1.55 $\pm$ 0.21                                                         | 1.22 $\pm$ 0.20                                                         | 1.56 $\pm$ 0.32                                                          |
| 31,000                   | 1.12 $\pm$ 0.14                                                         | 1.23 $\pm$ 0.18                                                         | 0.91 $\pm$ 0.16                                                          |
| 29,000                   | 0.89 $\pm$ 0.11                                                         | 1.24 $\pm$ 0.17                                                         | 1.15 $\pm$ 0.41                                                          |
| 24,000                   | 1.12 $\pm$ 0.17                                                         | 0.90 $\pm$ 0.15                                                         | 1.10 $\pm$ 0.18                                                          |
| 22,000                   | 1.04 $\pm$ 0.10                                                         | 1.16 $\pm$ 0.14                                                         | 0.76 $\pm$ 0.07                                                          |
| 20,000                   | *2.16 $\pm$ 0.28                                                        | 1.13 $\pm$ 0.10                                                         | 1.18 $\pm$ 0.14                                                          |

SDS gel electrophoresis and autoradiography of eye samples from individual *Hermisenda* were conducted as described in Fig. 1 legend. The autoradiograms were scanned with a Zeineh soft-laser densitometer and the phosphoprotein bands quantified by measuring peak heights above background by a procedure similar to those described elsewhere<sup>38-40</sup>. The validity of the procedure for the conditions of autoradiography used here was confirmed by comparing the data with that obtained by measuring radioactivity in 2-mm sections of sample lanes by liquid scintillation counting ( $r = 0.949$ ). The data obtained by the peak height method also compared favourably with that obtained by measuring the area under clearly separated peaks ( $r = 0.944$ ). The peak height method was routinely used because it allowed estimation of phosphoproteins not completely separated by electrophoresis. Relative levels of phosphoproteins were based on total phosphate incorporated into 11 bands detected in eyes from all four groups of animals. Eye samples from untreated animals and the experimental group were analysed on the same gel. Ratios of experimental to untreated were determined for each band from eye samples of individual animals. The data summarize four replications of the paired group (total  $n = 16$ ), three replications of the random group (total  $n = 12$ ) and one group receiving unpaired stimuli ( $n = 5$ ).

\*  $F_{2,30} = 6.49$ ;  $P < 0.01$ .



**Fig. 2** Densitometric scans of the 25,000–20,000-MW region of autoradiograms of eye samples obtained from animals receiving paired or random light and rotation. Autoradiograms were prepared as described in Fig. 1 legend. A 2–3-day exposure yields a linear relationship between radioactivity and grain density for phosphoprotein bands representing  $\leq 10\%$  of the total phosphoproteins. Autoradiograms were scanned with a Zeineh soft-laser densitometer. Absorbance is in arbitrary units. The densitometric scans are from the following samples in Fig. 1: —, paired: lane 4; ----, random: lane 1.

light paired with rotation has been attributed to light-induced changes in voltage-dependent  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  conductances<sup>13</sup>. The input resistance and the long-lasting depolarization can be enhanced by intracellular injection of the catalytic subunit of protein kinase into the photoreceptors<sup>41</sup>. It seems reasonable to suggest that the change in the level of the 20,000-MW phosphoprotein band in the eyes of trained animals is related to the known changes in the electrophysiological properties of type-B photoreceptors, but the unlikely possibility that the change is located elsewhere in the eye cannot be excluded.

Among the many possible biochemical processes that may be involved in behavioural modification, post-translational protein modification offers several attractive features. The enzyme-catalysed modifications can occur rapidly, would be likely to exhibit a high degree of specificity, are reversible and have the potential for amplifying an extracellular stimulus. Protein phosphorylation–dephosphorylation, as catalysed by protein kinases and phosphatases respectively<sup>23,24</sup>, is probably the most extensively studied post-translational protein modification in the nervous system (for review see ref. 25). Greengard<sup>26</sup> has discussed the possible roles of phosphorylated proteins in neuronal functions and has speculated that protein phosphorylation–dephosphorylation may be one of the biochemical mechanisms by which short-term and long-term information storage and retrieval are regulated. It has also been shown that changes in brain phosphoprotein bands follow electrical stimulation of nerve pathways in hippocampal slices known to produce long-term changes in synaptic activity<sup>27,28</sup>, and transient changes in the phosphorylation of cortical membrane proteins have been reported after electroconvulsive shock<sup>29</sup>. For marine invertebrates, neuronal protein kinases have been reported in *Aplysia*<sup>30-34</sup>, *Loligo*<sup>35</sup> and *Hermisenda*<sup>15</sup>. Electrical stimulation<sup>32,35</sup>, cyclic nucleotides and their analogues<sup>30-34</sup> and neurotransmitters<sup>30,33</sup> have been shown to alter levels of neuronal phosphoproteins and protein kinase. Serotonin and cyclic AMP can mimic the presynaptic facilitation of post-synaptic potentials in *Aplysia* motor neurones which underlie sensitization of the gill-withdrawal reflex<sup>36</sup>, and can stimulate

phosphorylation of a membrane phosphoprotein band in abdominal ganglia<sup>33</sup>, suggesting a role for protein phosphorylation in short-term sensitization.

Protein phosphorylation is clearly involved in associative learning in *Hermisenda*. The change in the 20,000-MW phosphoprotein band is highly specific because (1) 10 other phosphoprotein bands in the same sensory structure were not affected; and (2) the alteration was observed in eyes from animals presented with paired, but not random, stimuli. Several possible mechanisms could explain the change. The pairing of stimuli could activate or induce a protein kinase which catalyses the phosphorylation of the 20,000-MW protein. Alternatively, a phosphatase may be activated or induced during acquisition, resulting in the removal of phosphate group(s) from the 20,000-MW protein, which would then be phosphorylated by protein kinase. Another possibility is that acquisition could increase the rate of synthesis of a 20,000-MW acceptor protein. Although none of these possibilities can yet be excluded, the activation of protein kinase may be involved in the increase in the 20,000-MW phosphoprotein band because injection of the catalytic subunit of protein kinase into type-B photoreceptors of untreated animals mimics the effects of associative learning on the electrophysiological properties of these photoreceptors<sup>41</sup>.

It will be of interest to examine vertebrate models of associative learning to determine whether protein phosphorylation is related to learning in animals with more complex nervous systems.

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## Dissociation of platelet activation from transmethylation of their membrane phospholipids

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In cell membranes, two different methyltransferases catalyse the conversion of phosphatidylethanolamine (PE) into phosphatidylcholine (PC) using *S*-adenosylmethionine as methyl donor. This methylating process may affect various membrane functions. Thus, in erythrocytes, the accumulation of phosphatidyl-*N*-monomethylethanolamine, the product of the first methylation step of PE, increases membrane fluidity<sup>1</sup> and  $\text{Ca}^{2+}$ -ATPase activity<sup>2</sup>. In addition, methylation of PE in various cell types is increased when different agonists interact with their receptors leading to changes in calcium flux<sup>3</sup> and in adenylate cyclase and phospholipase A<sub>2</sub> activities<sup>4,5</sup>. However, the existence of a causal relationship between methylation of phospholipids and cell stimulation is questionable and here we present evidence against such a relationship in the case of the platelets stimulated to aggregate.

The role of phospholipid methylation was investigated using an inhibitor of methylation, 3-deazaadenosine ( $\text{C}_3\text{ado}$ ), and L-homocysteine thiolactone (L-HCys), which potentiates the inhibiting effects of  $\text{C}_3\text{ado}$ <sup>3</sup>.  $\text{C}_3\text{ado}$  and L-HCys (used at 0.1 and 0.5 mM respectively unless otherwise stated) are substrates for *S*-adenosylhomocysteine hydrolase which yields 3-deazaadenosylhomocysteine, a potent inhibitor of various methyltransferases<sup>6–9</sup>. Blood from male Wistar rats (~400g) was collected from the cannulated carotid artery into plastic tubes containing heparin (10 U per ml blood). Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 200g for 10 min and the concentration of platelets was adjusted to  $5 \times 10^8 \text{ ml}^{-1}$  with platelet-poor plasma. The incorporation of  $^3\text{H}$ -methyl into the platelet phospholipids was measured as described in Fig. 1 legend. In some experiments, the phospholipids were fractioned on silica gel TLC plates (Merck 60 F254) using chloroform/propionic acid/propanol/water (1:2:2:1 by vol) as solvent. Seventy-five per cent of the total radioactivity was found in the phospholipids which co-chromatographed with pure phosphatidylmonomethylethanolamine, phosphatidyl dimethylethanolamine and PC. The incorporation of  $^3\text{H}$ -methyl into phospholipids was linear with time for at least 6 h. When the incorporation was performed at 22 °C, methylation was completely suppressed by  $\text{C}_3\text{ado}$  alone or  $\text{C}_3\text{ado}$  plus L-HCys and almost totally suppressed by L-HCys alone (Fig. 1). In two experiments at 37 °C, L-HCys failed to prevent methylation of the phospholipids whereas  $\text{C}_3\text{ado}$  blocked as much as at 22 °C.

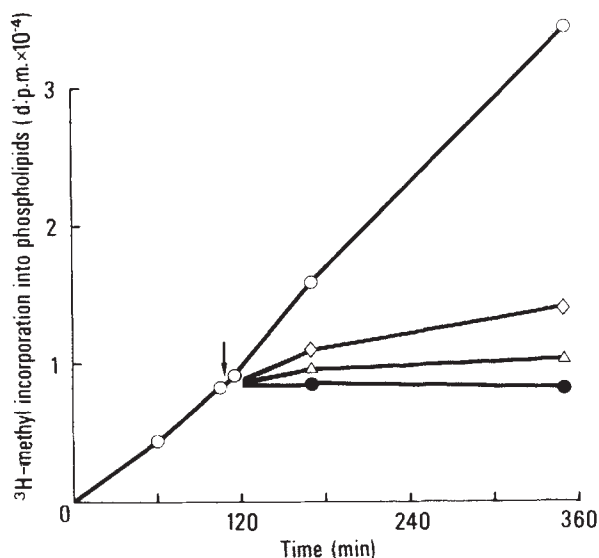
Platelet aggregation by collagen ( $5 \mu\text{g ml}^{-1}$ ) was reproducible for at least 4 h in control PRP. In contrast, when  $\text{C}_3\text{ado}$  and L-HCys were incubated together at 22 °C with PRP, aggregation decreased rapidly during the first 2 h of incubation, platelets becoming refractory to  $5 \mu\text{g ml}^{-1}$  collagen (Fig. 2). Neither  $\text{C}_3\text{ado}$  nor L-HCys used alone interfered with platelet aggregation. Inhibition was overcome when  $25 \mu\text{g ml}^{-1}$  of collagen were used. The release of ATP, measured as described in Fig. 3 legend, was reduced in parallel with aggregation. When threshold concentrations of ADP ( $0.4 \mu\text{M}$ ) or of convulxin ( $0.10 \mu\text{g ml}^{-1}$ ), a highly purified glycoprotein extracted from the venom of *Crotalus durissus cascavella* with specific aggregating ability<sup>10,11</sup>, were used as agonists on platelets incubated for 4 h with  $\text{C}_3\text{ado}$  and L-HCys, marked differences were observed: the



aggregation and release triggered by convulxin were suppressed whereas ADP-induced aggregation was only marginally affected (Fig. 3).

Because L-HCys interfered with phospholipid methylation at 22 °C, but not at 37 °C, aggregations were also studied after incubation of the platelets with the inhibitors at 37 °C. At this temperature C<sub>3</sub>ado associated with L-HCys did not inhibit aggregation induced by collagen (5 µg ml<sup>-1</sup>). This may be due to more rapid degradation of L-HCys at 37 °C than at 22 °C, L-HCys being rapidly catabolized within the cell<sup>12</sup> whereas C<sub>3</sub>ado is reported not to be deaminated<sup>7</sup>.

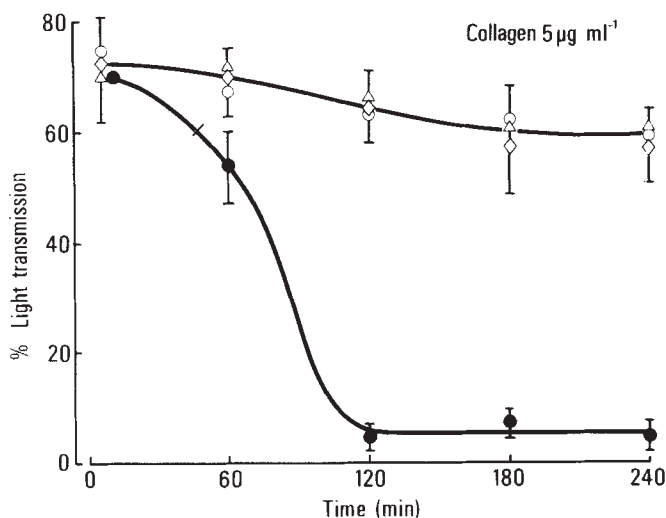
To investigate further the anti-aggregating effect of inhibitors of methylation, C<sub>3</sub>ado was used at 1 mM concentration. When L-HCys (0.5 mM) and C<sub>3</sub>ado (1 mM) were added together to PRP at 22 °C, the effects of collagen and convulxin were suppressed even with the higher concentrations used, whereas the effects of only the lowest concentrations of ADP used were partially inhibited. Finally, aggregation by the calcium ionophore A23187 (ref. 13) was unaffected, demonstrating that direct entry of Ca<sup>2+</sup> bypasses the suppressive effects of the combined inhibitors (Fig. 3).



**Fig. 1** Interference of 3-deazaadenosine and L-homocysteine with methylation of platelet phospholipids. Rat PRP ( $5 \times 10^8$  platelets ml<sup>-1</sup>) was incubated at 22 °C with 160 µCi ml<sup>-1</sup> of [*Me*-<sup>3</sup>H] L-methionine (2 µM, 80 Ci mmol<sup>-1</sup>, NEN) for 110 min and then divided into four aliquots (black arrow) which were incubated for 4 more hours with:  $\diamond$ , 0.5 mM L-HCys (Sigma);  $\Delta$ , 0.1 mM C<sub>3</sub>ado (Southern Research Institute);  $\bullet$ , 0.1 mM C<sub>3</sub>ado + 0.5 mM L-HCys; or  $\circ$ , the same volume of saline. At each indicated interval, duplicate 350-µl samples were collected and the reaction stopped by addition of 2 ml of ice-cold buffer (15 mM Tris-HCl, 134 mM NaCl, 10 mM EDTA, 10 mM L-methionine, pH 7.4). The platelets were separated from plasma by centrifugation and washed once with the same buffer. After addition of trichloroacetic acid (10%, 2 ml), the pellet was centrifuged (30,000g, 10 min) and the phospholipids extracted with 3 ml chloroform/methanol (2:1, v/v) and then washed twice with 2 ml of a 50% methanol/0.1 M KCl solution. The methylation of the phospholipids was measured after drying 1 ml of the chloroform phase and adding the scintillation mixture. The data shown are typical of three separate experiments.

To examine the possible involvement of the adenylate cyclase system with the activity of C<sub>3</sub>ado and L-HCys<sup>8</sup>, the effect of prostaglandin (PG) E<sub>2</sub> (Sigma) was studied. When a pro-aggregatory dose of PGE<sub>2</sub> (1 µM) was added to rat PRP 1 min before a submaximal amount of ADP or of collagen, aggregation of control platelets and of those incubated for 4 h with C<sub>3</sub>ado and L-HCys (1 and 0.5 mM respectively) was potentiated to a similar extent (~60%). As PGE<sub>2</sub> potentiates aggregation by reducing the platelet adenylate cyclase activity<sup>14</sup>, we conclude that the latter is unaffected by the combined activity of C<sub>3</sub>ado and L-HCys.

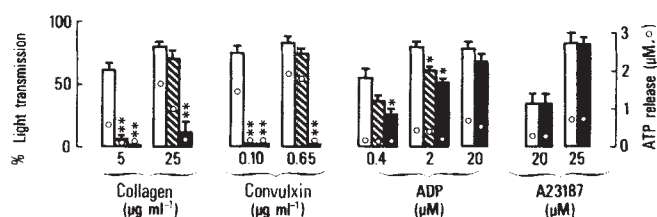
Our results demonstrate that methylation of PE to give PC occurs in platelets as previously reported<sup>15,16</sup> and is suppressed



**Fig. 2** Time-dependent inhibition by 3-deazaadenosine and by L-homocysteine of collagen-induced aggregation. Rat PRP was obtained as described in the text and incubated at 22 °C with 0.5 mM L-HCys ( $\diamond$ ); 0.1 mM C<sub>3</sub>ado ( $\Delta$ ); 0.1 mM C<sub>3</sub>ado + 0.5 mM L-HCys ( $\bullet$ ); or saline ( $\circ$ ). At each indicated time (5, 60, 120, 180, 240 min) 0.4-ml samples were withdrawn. Aggregations induced by collagen (5 µg ml<sup>-1</sup>) were studied at 37 °C under constant stirring at 1,100 r.p.m. Each point is the mean  $\pm$  s.e.m. ( $n = 5$ ) of the maximum aggregation, expressed as per cent of light transmission.

by known inhibitors of this pathway. Aggregation and release of ATP due to collagen and to the novel aggregating agent convulxin were also inhibited by the association of C<sub>3</sub>ado and L-HCys, in conditions where the platelet aggregating mechanisms were preserved, as shown by the maintained responsiveness to ADP and to the ionophore A23187. Nevertheless, a marked dissociation was noted between the inhibition of phospholipid methylation and that of platelet activation: the former showed a rapid onset whereas the latter occurred after ~2 h of incubation. On the other hand, C<sub>3</sub>ado (0.1 mM) by itself rapidly blocked methyl transfer to phospholipids while it did not alter platelet aggregation over a 4-h incubation period. Hence the inhibition of phospholipid methylation does not seem to be directly responsible for the platelet suppressive effects of C<sub>3</sub>ado and L-HCys. Furthermore, we failed to demonstrate an increase of the methyl incorporation into phospholipids when platelets were stimulated with 25 µg ml<sup>-1</sup> of collagen, measurements being performed within 0.5, 1, 2, 10 and 30 min. We conclude that platelet activation does not require increased biosynthesis of PC from PE during agonist-receptor interaction.

A potential target for the methylation inhibitors might be a protein needed for platelet aggregation. In other cells, protein carboxymethylation has proved crucial in chemotaxis<sup>17</sup> and neurosecretion<sup>18</sup>. Whatever the explanation for the inhibition of platelet function, blockade by C<sub>3</sub>ado, associated with L-HCys, of collagen- and convulxin-induced aggregation and the very



**Fig. 3** Differential inhibition by 3-deazaadenosine and L-homocysteine of platelet activation induced by collagen, convulxin, ADP and A23187. Rat PRP was incubated for 4 h at 22 °C with 0.1 mM C<sub>3</sub>ado + 0.5 mM L-HCys ( $\blacksquare$ ), 1 mM C<sub>3</sub>ado + 0.5 mM L-HCys ( $\blacksquare$ ), or saline ( $\square$ ) before challenge with collagen (Horn Hormon Chemie), convulxin, ADP (Sigma) or A23187 (Boehringer-Mannheim). Results are mean  $\pm$  s.e.m. (4–8 experiments) of the maximum of aggregation as measured as per cent of light transmission. The statistical significance of the differences between control and treated platelets was evaluated by the paired Student's *t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ . Release of ATP ( $\circ$ ) was measured in 20-µl samples collected 1 min after the addition of the aggregating agents with the luciferin-luciferase technique<sup>10</sup>.

weak effect against ADP distinguishes two families of platelet-stimulating agent. It is interesting that convulxin and collagen are potent activators of rat platelet phospholipase A<sub>2</sub>, as measured by thromboxane B<sub>2</sub> production, whereas ADP is almost inactive (in preparation). This suggests that methylation inhibitors act at a site closely related to the membrane modification which lead to phospholipase A<sub>2</sub> activation.

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**Note added in proof:** Some of these results have been confirmed recently by Hotchkiss *et al.*<sup>19</sup>.

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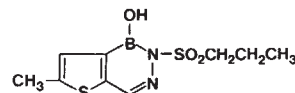
## A diazaborine derivative inhibits lipopolysaccharide biosynthesis

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**Inhibitors of the biosynthesis of peptidoglycan, the rigid layer of the bacterial cell wall which is responsible for the shape of the cell, are among the most effective antibacterial agents known. Both Gram-positive and Gram-negative organisms are susceptible to the action of such compounds. As lipopolysaccharide (LPS) is an integral part of the outer membrane of Gram-negative bacteria, it is possible that a blockage of its biosynthesis might result in the arrest of cellular growth and eventually cell death, analogous to the effects seen with inhibitors of peptidoglycan biosynthesis. However, no antibacterial agent has been described which inhibits growth in this way. We present here experimental evidence which indicates that 1,2-dihydro-1-hydroxy-6-methyl-2-(propanesulphonyl)-thieno(3,2-D)(1,2,3)-diazaborine (code no. 84474), a heterocyclic, boron-containing substance, prevents bacterial proliferation by inhibiting LPS biosynthesis. There was a reduction in galactose incorporation into the LPS of whole cells, and no newly formed LPS chains appeared in whole bacteria in the presence of the compound, as demonstrated by electron microscopy. The 2-keto-3-deoxy-octonate metabolism seems to be the target of 84474 as arabinose 5-phosphate incorporation into macromolecular material was affected.**

Diazaborines are synthetic compounds, having one boron and two nitrogen atoms in a six-membered ring<sup>1–3</sup>. Most show moderate to good antibacterial activity. Analysis of the antibacterial spectrum of diazaborines synthesized in our laboratories showed that only Gram-negative organisms were affected, suggesting that these antibacterials might interfere with the biosynthesis of a specific component of such bacteria. To investigate this we used 84474 (see formula below) as a model compound, assuming that inhibition of LPS biosynthesis by this substance is a general property of diazaborines.



The compound was tested using *Escherichia coli* K-12 and *Salmonella typhimurium* mutants. The MIC (minimum inhibitory concentration) values, determined by the tube dilution method, were 1.25 and 2.5 µg ml<sup>-1</sup> for *E. coli* and *Salmonella*, respectively. *E. coli* K-12 has a rough colony morphology, that is, it produces LPS molecules that lack the O-antigenic side chains. The LPS molecule contains one galactose residue in a 1,6 branch of the core oligosaccharide chain<sup>4,5</sup>. As LPS is the only macromolecular structure in these bacteria that contains galactose, we used the incorporation of galactose into whole *E. coli* cells as an index of LPS biosynthesis. A galactose-epimerase-negative mutant of *E. coli* K-12, PL2, was chosen for this experiment to avoid the spread of the radioactive label, introduced via galactose, into the general carbon metabolism of the bacteria.

As shown in Fig. 1, the incorporation of galactose into macromolecular material was significantly inhibited by very low concentrations of the diazaborine. Other antibacterials with different modes of action, such as chloramphenicol, epicillin, mecillinam and bicyclomycin, showed no such effects (Table 1). Interestingly, all β-lactam antibiotics tested enhanced galactose incorporation; the reason for this is unknown. However, transport of galactose into bacterial cells was not inhibited by the diazaborine. This was shown by centrifuging the bacteria, after incubation with <sup>3</sup>H-galactose, through a layer of silicone oil and by measuring the radioactivity in the cell pellet<sup>6</sup>. Both with and without diazaborine, the bacterial cells accumulated similar amounts of galactose (data not shown). The step which is sensitive to the inhibitor must therefore be the incorporation of labelled galactose into macromolecular material. The incorporation of galactose into LPS was demonstrated by phenol extraction of *E. coli* cells after incubation in radiolabelled galactose-containing medium by the method of Westphal and Jann<sup>7</sup>, and subsequent SDS-polyacrylamide gel electrophoresis of the aqueous phase. About 75% of the radioactivity migrated on the gel with the mobility of a purified, marker LPS. Some radioactivity was associated with an unidentified, low-molecular weight compound, which migrated with the bromophenol marker.

The inhibition of LPS biosynthesis by diazaborine was also demonstrated by electron microscopy. We prepared antibodies against *S. typhimurium* SF1135, a wild-type strain which

**Table 1** Incorporation of <sup>3</sup>H-galactose in *E. coli* PL2

|                         | Concentration<br>(µg ml <sup>-1</sup> ) | c.p.m.* |
|-------------------------|-----------------------------------------|---------|
| Control (no antibiotic) |                                         | 73,398  |
| 84474                   | 20                                      | 3,304   |
| Chloramphenicol         | 20                                      | 71,750  |
| Epicillin               | 10                                      | 126,374 |
| Mecillinam              | 20                                      | 121,410 |
| Bicyclomycin            | 20                                      | 91,255  |

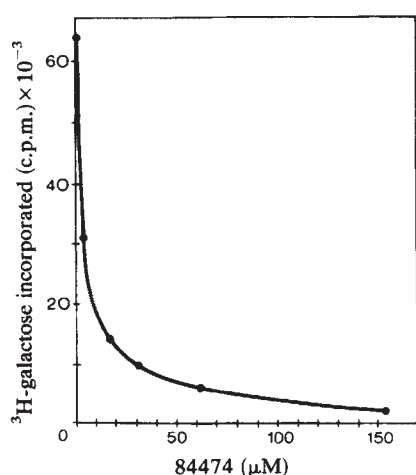
The basic procedure was as described in Fig. 1 legend except that time of incubation with antibiotic was 30 min.

\* Mean of two independent experiments.

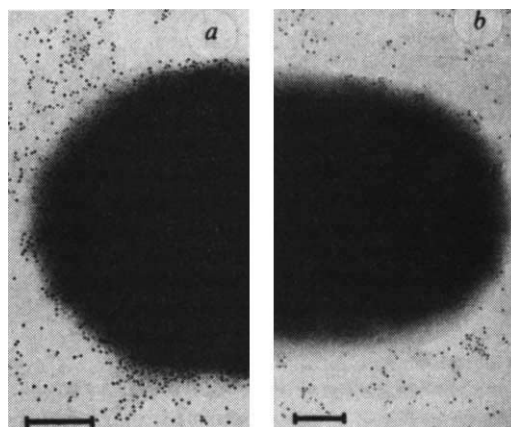


contains a smooth LPS having complete O-antigenic polysaccharide chains. The binding of these antibodies to the O-antigenic chains of the wild-type cells was visualized by labelling with ferritin as described elsewhere<sup>8</sup>. No binding occurred if the corresponding galactose-epimerase-negative mutant, SF1195, was mixed with the ferritin-labelled antibodies. This was expected, as the *Salmonella* strain used had been grown in the absence of galactose and was therefore unable to incorporate this sugar into the core oligosaccharide of LPS; thus it produced an incomplete, 'rough' LPS molecule. Mührladt *et al.*<sup>8</sup> showed that addition of galactose to the growth medium of these mutants leads, within minutes, to the appearance of the complete, 'smooth' LPS on the cell surface and it can be detected by binding of the ferritin-labelled antibodies. By this method, we were able to determine the kinetics of LPS biosynthesis and monitor its appearance on the outer cell wall of the bacteria. The synthesis of complete LPS molecules was strongly inhibited when *Salmonella* galE<sup>-</sup> cells were treated with diazaborine for a short time before the addition of galactose to the medium. The electron microscopic images of control and 84474-treated *Salmonella* cells, visualized by binding of the ferritin-labelled antibodies, are shown in Fig. 2. A distinct reduction in the number of newly formed 'smooth' LPS chains was detected when the dose of inhibitor was  $>10 \mu\text{g ml}^{-1}$ . None of the standard antibiotics investigated in this system (chloramphenicol, cephaloridine, epicillin and bicyclomycin), each applied at  $50 \mu\text{g ml}^{-1}$ , gave any detectable alteration in the amount of antibody bound per cell. A modification (N. v. Jeney, personal communication) of the ELISA method<sup>9</sup> gave quantitative results that corroborated the electron microscopic observations. With this test, inhibition could be observed at concentrations of diazaborine as low as  $2 \mu\text{g ml}^{-1}$  (data not shown).

The O-antigenic polysaccharide portion, which is linked to the LPS 'core', can be lost due to mutation, without any apparent deleterious effect on the cell<sup>10</sup>. Cells having LPS in which the oligosaccharide chain is even less extended as a result of a mutation are still viable, the most drastic changes being in the 'Re' mutants. These produce only a truncated LPS without any sugars attached to the 2-keto-3-deoxy-octonic acid (KDO) moieties. Some of these 'deep rough' mutants show limitations in their growth behaviour in certain media<sup>10</sup>. The existence of such viable mutants suggests that inhibition by a drug of the extension of the polysaccharide part of LPS would not be a lethal event. However, the mutationally induced blockage in the



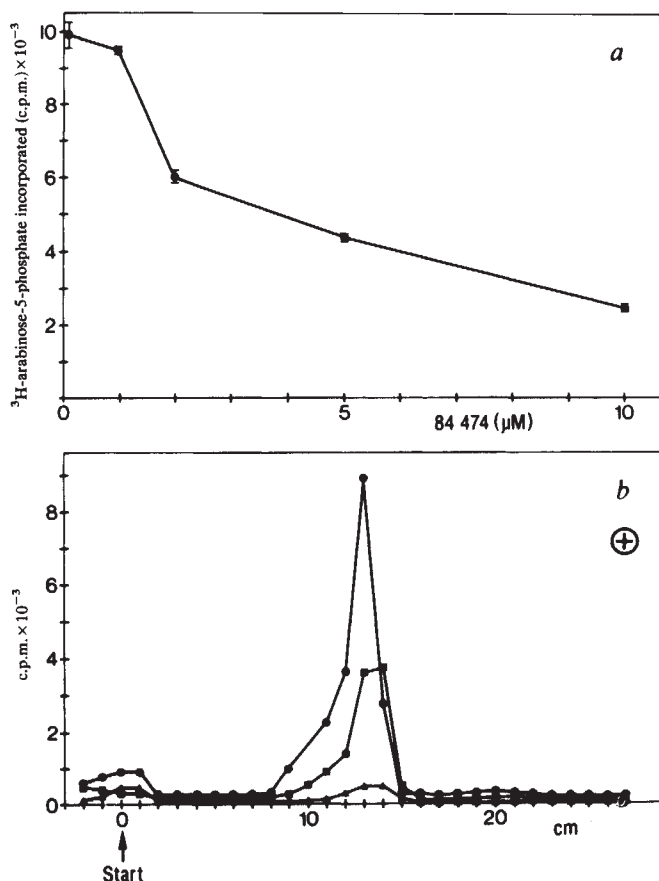
**Fig. 1** Incorporation of  $^3\text{H}$ -galactose in *E. coli* PL2. *E. coli* PL2 (galE<sup>-</sup>), a mutant originally described by Buttin<sup>16</sup> (obtained from the *E. coli* Stock Center, New Haven), was grown at 37 °C in a synthetic M9 medium<sup>17</sup> containing 0.5% glycerol as a carbon source for the early logarithmic phase ( $A_{600} = 0.3$ ), at which point  $1 \mu\text{Ci ml}^{-1}$   $^3\text{H}$ -galactose ( $9.3$  or  $22.3 \text{ Ci mmol}^{-1}$ ) was added. Growth was continued for 10 min and 0.5-ml portions were filtered on to Whatman GF/C filter disks. The filters were washed with 100 ml boiling water, dried and counted after addition of a toluene-based scintillation fluid.



**Fig. 2** The bacterial strains used here were obtained from G. Schmidt, Max-Planck-Institut für Immunologie, FRG. *S. typhimurium* SF 1195 (galE<sup>-</sup>) was grown in several flasks under aeration in M9 medium with glycerol to the late logarithmic phase ( $A_{600} = 1$ ). At this point various quantities of 84 474 were added to give 2, 10, 50 and  $100 \mu\text{g ml}^{-1}$  final concentrations of the diazaborine. One sample contained no inhibitor. After further incubation for 5 min galactose was added to a final concentration of 50 mM. The cultures were incubated for another 5 min then the bacteria were collected by centrifugation and washed once with saline. After centrifugation the cell pellet was suspended in 200  $\mu\text{l}$  of a ferritin-labelled anti-SF1135 IgG solution in 0.89% NaCl/30 mM sodium barbital buffer, pH 7.2, and left in an ice bath for 1 h. The ferritin-labelled antibody was prepared according to Mührladt *et al.*<sup>8</sup>. After incubation, the bacterial cells were pelleted by centrifugation, washed with the NaCl-containing barbital buffer, resuspended in the same medium and applied to formvar-carbon-layered copper grids. *a*, *Salmonella* SF1195 without antibiotic treatment and *b*, after pretreatment with  $50 \mu\text{g ml}^{-1}$  84 474. Scale bars, 200 nm.

synthesis of KDO, which is incorporated as a trisaccharide into the lipopolysaccharide chain, seems to result in non-viable variants<sup>11</sup>. We therefore tested for interference with KDO metabolism or utilization by 84474. Metabolism of KDO was monitored by allowing the cells to use radioactive D-arabinose 5-phosphate, a precursor in the synthesis of KDO which can be actively transported into bacterial cells via the hexose monophosphate transport system<sup>11</sup>. This uptake in *S. typhimurium* AG 701 (*uhp*<sup>+</sup>) was efficient and was not disturbed by  $10 \mu\text{M}$  diazaborine. This was shown by washing the bacteria with physiological saline at room temperature after incubation with labelled arabinose 5-phosphate and 84474. Both control and drug-treated bacteria retained an equal amount of radioactivity at various time intervals (data not shown). However, incorporation into material that could not be extracted from the cells by hot water treatment, for example, LPS and other macromolecular compounds, was strongly inhibited (Fig. 3a). Fifty per cent inhibition occurred in the micromolar concentration range of the inhibitor.

KDO is formed from D-arabinose 5-phosphate and phosphoenol pyruvic acid via an 8-phosphorylated intermediate by the action of KDO 8-phosphate synthetase, and is subsequently dephosphorylated by the enzyme KDO 8-phosphate phosphatase<sup>12,13</sup>. KDO is then 'activated' by the CMP-KDO-synthetase, with CTP as second substrate, to give CMP-KDO. From this intermediate, KDO is linked to the 'precursor molecule' by the membrane-bound CMP-KDO-transferase<sup>14</sup>. It is expected that inhibition of one of the enzymes involved in KDO metabolism by diazaborine would result in an accumulation of the substrate of this particular enzyme. We demonstrated this by culturing *S. typhimurium* AG 701 with  $^3\text{H}$ -D-arabinose 5-phosphate for several hours to avoid pool effects and by adding diazaborine in the last 30 min of the incubation period; there was an elevated KDO content. This was shown by treating the



**Fig. 3** Incorporation of  $^3\text{H}$ -D-arabinose 5-phosphate. *a*, *S. typhimurium* AG 701 (from P. Ray, Burroughs Wellcome) was grown in 50 ml of M9 medium with glucose. When the bacterial growth was in the early log phase, the culture was subdivided into 2-ml fractions,  $0.5 \mu\text{Ci } ^3\text{H}$ -D-arabinose 5-phosphate ( $42.5 \text{ mCi mmol}^{-1}$ )<sup>18</sup> per ml was added, and the incubation continued for 15 min. The inhibitor was added at various concentrations together with D-arabinose 5-phosphate. The samples were filtered through nitrocellulose filters and washed repeatedly with 3 ml of boiling water. The filters were dried and counted in a toluene-based scintillation fluid. *b*, When the intracellular radioactivity of low-molecular weight material was analysed, the time of labelling of the cells was different. The bacteria were grown continuously in the presence of  $^3\text{H}$ -D-arabinose 5-phosphate ( $49 \text{ mCi mmol}^{-1}$ ), then collected on nitrocellulose filters, washed twice with cold water and several times with boiling water. The hot water fractions were collected separately, evaporated to small volumes under reduced pressure, applied to Whatman 3MM paper and separated by high-voltage electrophoresis in 0.05 M ammonium formate, pH 2.9, at  $50 \text{ V cm}^{-1}$  for 60–90 min. Picric acid and authentic KDO served as reference compounds. The radioactivity on the paper was determined by cutting the paper into 1 cm-wide strips and counting the water eluate in a scintillation spectrometer. The reference KDO was detected by heating the paper to  $140^\circ\text{C}$  for 2 min—this produces a brightly fluorescing spot at the position of KDO in UV light (F. M. Unger, personal communication). The prominent radioactive peaks show the same mobility as authentic KDO.  $\Delta$ , Control, no additions;  $\blacksquare$ ,  $2 \mu\text{g ml}^{-1}$  84474;  $\bullet$ ,  $10 \mu\text{g ml}^{-1}$  84474.

bacteria with hot water and then analysing the filtrates by paper electrophoresis (Fig. 3b). The accumulation of KDO can be explained either by an inhibition of CMP-KDO-synthetase or by an accumulation of CMP-KDO, the last of the substrates located in the cytoplasm. Extraction of the highly unstable CMP-KDO<sup>14</sup> would invariably lead to the generation of KDO. When tested in our institute, there was no observed effect of the diazaborine on the activity of partially purified CMP-KDO synthetase (F. M. Unger and H. Grasmuk, personal communication). Thus, it is reasonable to assume that 84474 interferes with the correct transfer of CMP-KDO to the lipid A-precursor molecule. The conclusion that 84 474 affects KDO

metabolism is consistent with the observed intrinsic resistance of *Bacteroides* species to diazaborines; the LPS of *Bacteroides fragilis* is thought to lack KDO<sup>15</sup>.

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## Fraction of myosin heads bound to thin filaments in rigor fibrils from insect flight and vertebrate muscles

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The binding of myosin heads to actin in rigor striated muscle is affected by steric constraints imposed by the structure of the filament lattice and the mismatch of the helical periodicities of the thick and thin filaments<sup>1,2</sup>. In rabbit fibres, despite these steric constraints, at least 95% of the myosin heads are attached to actin<sup>3–5</sup>. It has been suggested<sup>1,2,6</sup> that not all the myosin heads in insect flight muscle may be able to bind to the thin filament in rigor conditions. Here we compare the fraction of heads bound in the rigor state in the flight muscle from the blowfly (*Sarcophaga bullata*) and in striated muscle from the frogs *Rana pipiens* and *Rana temporaria*, using a tryptic digestion technique<sup>4,7,8</sup>. We find that whereas at least 95% of the heads are bound tightly in the frog muscle, only 70% of the heads are bound in rigor *Sarcophaga* muscle.

The SDS gel patterns of tryptic digests of frog myofibrils (Fig. 1) showed the same general features as those of the corresponding digests of rabbit fibrils<sup>4</sup>. In the absence of pyrophosphate, myosin heavy chain was digested primarily to species of molecular weights 200,000 (200 K) and 26 K. Digestion in the presence of MgPP produced species of 160 K, 51 K and 26 K, with transient species of 200 K and 74 K. It has been shown previously<sup>4</sup> that these patterns result from tryptic digestion at two sites, 26 K and 74 K from the N-terminus of the myosin heavy chain, the 74 K site being protected when the head is bound to actin. The intensities of the 160 K and 51 K species

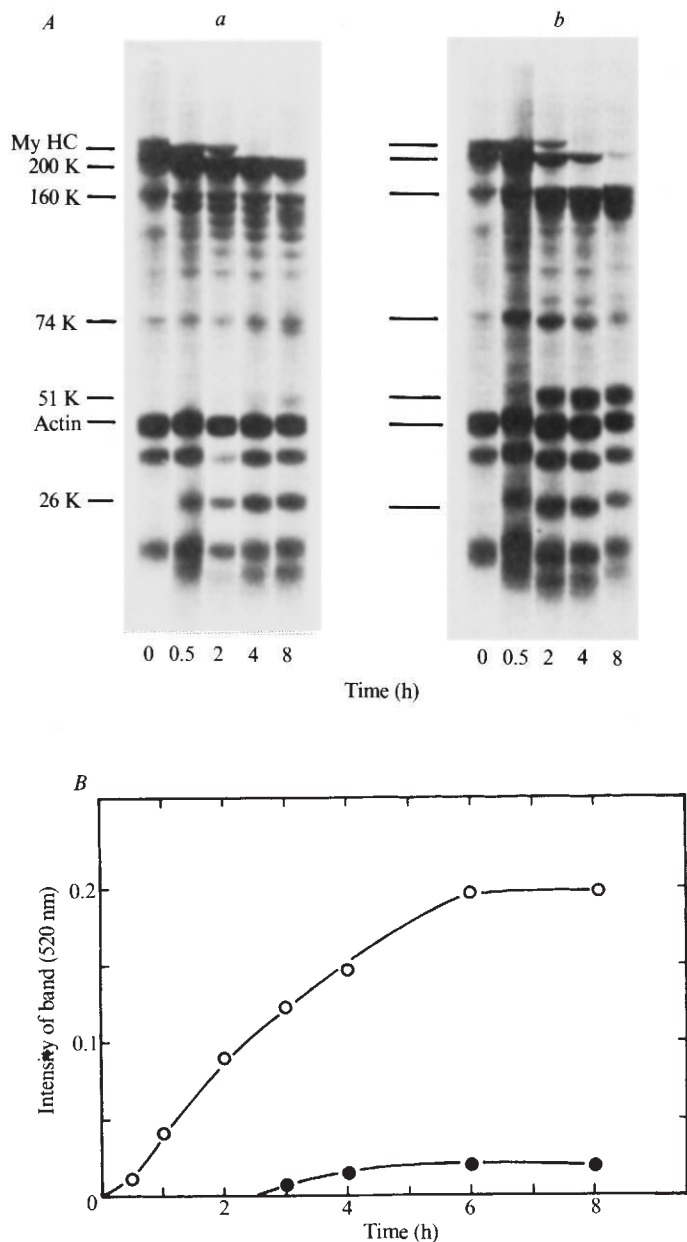
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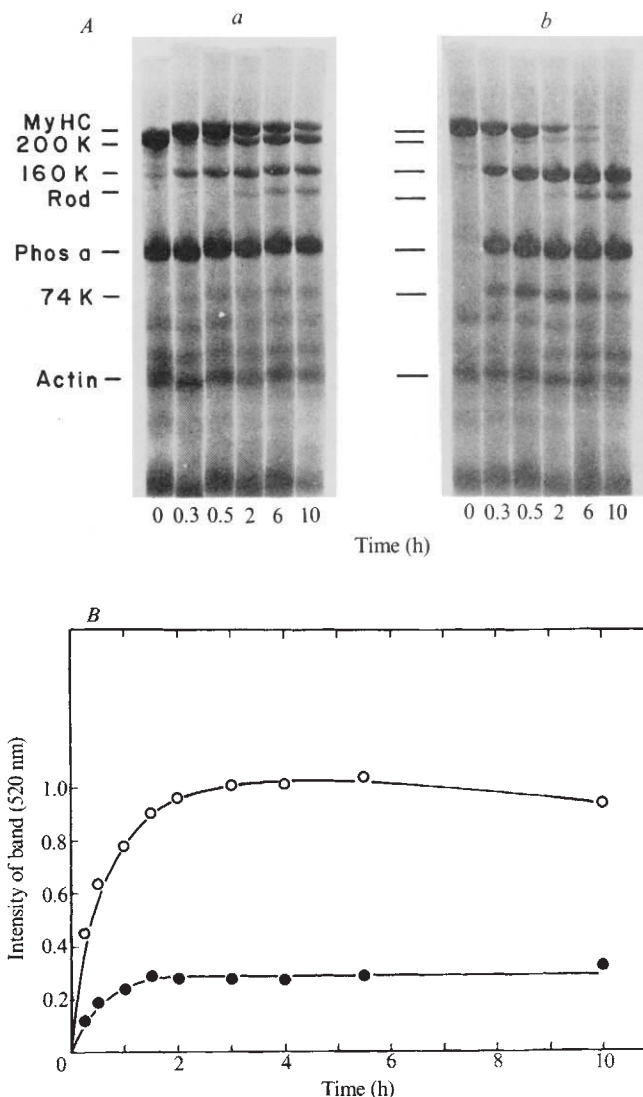
were both sensitive to the presence of  $\text{MgPP}_i$ . By monitoring the quantity of the 51 K species by the procedure described in Fig. 1 legend, we found that at least 95% heads were attached in fully overlapped rigor frog myofibrils. We observed very little tryptic cleavage in the rod portion of the frog myosin heavy chain in the

conditions described in Fig. 1 legend. Thus papain digestion of myofibrils previously digested with trypsin for 8 h produced 85–90% of the rod formed from control fibrils. This result was not influenced by the presence of  $\text{MgPP}_i$  during tryptic proteolysis.

Tryptic digestion of insect flight muscle fibrils also produced species of 200 K, 160 K and 74 K (Fig. 2), indicating that the trypsin cleavage sites 26 K and 74 K from the N-termini of vertebrate myosins are also present in insect myosin. After longer digestion times, small amounts of rod were produced. The presence of a 160 K species in digested rigor myofibrils indicates that not all heads are tightly bound to actin. The intensity of the 51 K species was not suitable for purposes of quantification because undigested insect fibrils already had a substantial quantity of 51 K– $M_r$  material. Gels of undigested



**Fig. 1** A, SDS gels showing the time course of tryptic digestion of frog myofibrils. *a*, Rigor; *b*, relaxed (+2 mM  $\text{MgPP}_i$ ). Myofibrils ( $2.0 \text{ mg ml}^{-1}$ ) were digested with trypsin ( $0.01 \text{ mg ml}^{-1}$ ) in buffer consisting of 20 mM imidazole-HCl, 0.1 M KCl, 4 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 1 mM EDTA, 1 mM dithiothreitol, pH 7.0 at  $5^\circ\text{C}$ . Digestion was terminated by addition of trypsin inhibitor to  $0.05 \text{ mg ml}^{-1}$  at the specified time. Myofibrils were prepared as described elsewhere<sup>4</sup>. Per cent overlap of heads with thin filaments was estimated to be 95. SDS polyacrylamide gel electrophoresis was carried out according to Weber and Osborn<sup>12</sup>. Gels contained 5% acrylamide–0.13% bis(acrylamide). MyHC, myosin heavy chain. B, time course of production of 51 K species. ●, Rigor; ○, relaxed (+2 mM  $\text{MgPP}_i$ ). SDS gels, stained with Coomassie brilliant blue G250, were densitometered at 520 nm on a Beckman Model DU monochromator equipped with a Gilford linear transport system and photomultiplier. The intensity of the 51 K species at each time point was estimated relative to the intensity of the actin band. Beer's law was obeyed by both actin and the 51 K species up to the sample loadings applied to these gels. The per cent heads attached to thin filaments in rigor fibrils was calculated from  $100[1 - I_{51K}(\text{rigor})/I_{51K}(\text{MgPP}_i)]$  where  $I_{51K}(\text{rigor})$  and  $I_{51K}(\text{MgPP}_i)$  are the intensities of the 51 K species after 6–8 h of tryptic digestion in rigor conditions and in the presence of 2 mM  $\text{MgPP}_i$ , respectively.



**Fig. 2** A, SDS gels showing the time course of tryptic digestion of myofibrils from insect flight muscle. *a*, Rigor; *b*, relaxed (+2 mM  $\text{MgPP}_i$ ). Myofibrils ( $0.5 \text{ mg ml}^{-1}$ ) were digested with trypsin ( $0.005 \text{ mg ml}^{-1}$ ) at  $5^\circ\text{C}$  in the buffer described in Fig. 1 legend. Digestion was quenched by the addition of trypsin inhibitor to  $0.05 \text{ mg ml}^{-1}$ . Phosphorylase *a* ( $0.1 \text{ vol at } 1 \text{ mg ml}^{-1}$ ) was added to the digests before denaturation with SDS. Myofibrils were prepared by the method of Reedy *et al.*<sup>10</sup>. It has been shown in the electron microscope<sup>10</sup> that the myosin heads are fully overlapped by thin filaments in fibrils from flight muscle of *S. bullata*. MyHC, myosin heavy chain. B, time course of production of 160 K species. ●, Rigor; ○, relaxed (+2 mM  $\text{MgPP}_i$ ). The intensity of the 160 K species ( $I_{160K}$ ) at each time was estimated relative to the intensity of the corresponding phosphorylase *a* band and corrected for the small amount of material present before digestion. The per cent heads attached to thin filaments in rigor conditions was calculated from  $100[1 - I_{160K}(\text{rigor})/I_{160K}(\text{MgPP}_i)]$  obtained after complete digestion of myosin heavy chain (4–10 h).

insect fibrils showed very little material migrating in the 160 K region and we therefore monitored formation of this species. Figure 2B shows the influence of  $MgPP_i$  on the production of the 160 K species and indicates that, in contrast to the vertebrate myofibrils, only 70% of the heads are attached tightly in rigor conditions. Three other digestion experiments yielded values of 66, 69 and 72 for the per cent heads tightly attached to actin.

Papain digestion of insect myofibrils previously treated with trypsin produced rod and S-1 (Fig. 3). Intact myosin heavy chain is present in these gels because purified myosin was added to the fibrils after papain digestion as a standard for densitometry of the gels. The results indicate that there was insignificant clipping within the rod by trypsin and therefore none of the 160 K species was produced by clipping in the myosin tails. The following experiment eliminates the possibility that 30% of the heads in the preparation of insect fibrils were denatured and therefore unable to bind to thin filaments. Native insect fibrils were digested with papain to give rod and S-1 and the total digest mixed with an equal volume of undigested insect fibrils to ensure that there was no shortage of actin. After centrifugation (100,000g, 2 h), SDS gels showed that >95% of the S-1 was in the pellet. If  $MgPP_i$  (5 mM) was included in the mixture, then only 5% of the S-1 was pelleted. Disorder of the filament lattice during preparation of the fibrils cannot account for the large fraction of unattached heads because there was no evidence of swelling, fraying or supercontraction of the fibrils under the phase-contrast microscope. The possibility that 30% of the heads in insect fibrils are susceptible to tryptic cleavage even when they are tightly bound to actin was also examined. We separated S-1 from papain-treated insect fibrils by a modification of the procedure of Cooke<sup>9</sup> and subjected mixtures of insect S-1 and rabbit actin to proteolysis with trypsin. In the presence of 5 mM  $MgPP_i$ , proteolysis of insect S-1/rabbit actin mixtures produced the 51 K species, whereas in rigor solvent conditions, insignificant (<5%) levels of the 51 K species was formed, showing that all the insect S-1 could be protected by actin. We conclude, therefore, that rigor insect flight muscle has a greater fraction (~30%) of unattached (or weakly bound) myosin heads than has vertebrate muscle.

Two types of model have been proposed to interpret the appearance of rigor insect flight muscle under the electron

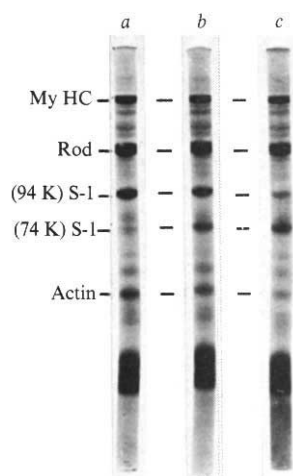
microscope. In one, each arm of the 'flared X' structure seen in transverse sections is ascribed to a separate myosin molecule<sup>1,2</sup>, whilst the alternative model<sup>6</sup> supposes that pairs of arms are contributed by the two heads of a single molecule. If the number,  $n$ , of myosin molecules at each 14.5-nm level is six<sup>10</sup>, then the percentages of attached myosin molecules for the two types of model have been estimated to be 67 and 37.5 respectively<sup>1,2,6</sup>, whereas if  $n = 4$  (ref. 11), these values would be 100 and 56 respectively. Our experimental determination that 70% of the heads are attached fits well with the first type of model if  $n = 6$ , but would require binding of only one head of about half the molecules if  $n = 4$ . The second model is clearly incompatible with our data if  $n = 6$ , but the discrepancy is much smaller if  $n = 4$ .

For both frog and rabbit we find that almost 100% of the heads are tightly attached. This result should prove useful in determining the extent to which a myosin head can move from its resting position on the thick filament in order to attach to actin.

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**Fig. 3** SDS gels of papain digests of *Sarcophaga* myofibrils. *a*, Native myofibrils ( $0.5 \text{ mg ml}^{-1}$ ); *b*, myofibrils ( $0.5 \text{ mg ml}^{-1}$ ) previously treated for 6 h with trypsin ( $0.005 \text{ mg ml}^{-1}$ ) in rigor conditions; *c*, myofibrils ( $0.5 \text{ mg ml}^{-1}$ ), previously treated for 6 h with trypsin ( $0.005 \text{ mg ml}^{-1}$ ) in the presence of 2 mM  $MgPP_i$ . Myofibrils ( $0.5 \text{ mg ml}^{-1}$ ) were digested with papain ( $0.05 \text{ mg ml}^{-1}$ ) for 90 min in the same conditions as in Fig. 1 legend. This time was sufficient for complete digestion of *Sarcophaga* myosin heavy chain (MyHC). Before denaturation of samples, 0.1 vol of rabbit myosin ( $1 \text{ mg ml}^{-1}$ ) was added. The amounts of rod were estimated relative to the amounts of the rabbit myosin heavy chain. Compared with the amount of rod in *a*, the amounts of rod in *b* and *c* were 103% and 98% respectively.

## Human cells contain two forms of pp60<sup>c-src</sup>

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Normal chicken embryo fibroblasts contain a gene, *c-src*, which is homologous to the avian sarcoma virus transforming gene, *src*<sup>1</sup>. Both genes code for a 60,000-molecular weight ( $M_r$ ) protein which is phosphorylated at serine and tyrosine residues<sup>2-11</sup> and has the unusual function of phosphorylating tyrosine residues<sup>10,11</sup>. We report here that both normal human fibroblasts and A431 human epidermoid carcinoma cells contain two forms of the normal cell protein pp60<sup>c-src</sup> which were resolved electrophoretically using a 10% polyacrylamide gel. The 60,000- $M_r$  form is phosphorylated primarily at tyrosine, while the 59,000- $M_r$  form is phosphorylated at serine. <sup>35</sup>S-methionine chymotryptic maps show that the two forms are clearly related, although several significant differences are observed.

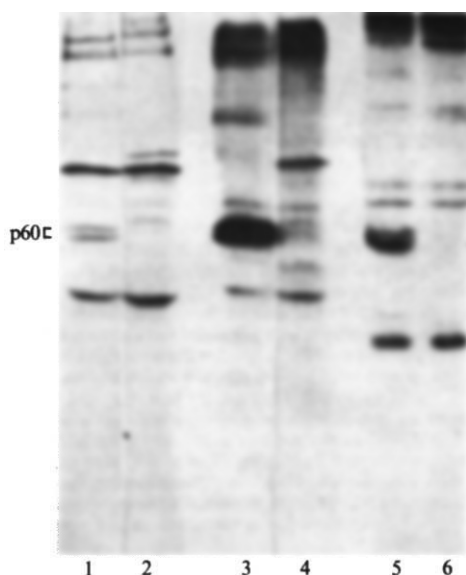
Avian sarcoma virus infection of normal avian cells leads to 50-100-fold greater synthesis of pp60<sup>c-src</sup> than normal and to increased levels of phosphotyrosine in total cell protein<sup>11</sup>. These events correlate quite well with the process known as transformation. Knowledge of the normal function of pp60<sup>c-src</sup> might help to elucidate the mechanism of viral transformation.

A431 cells have an elevated concentration of epidermal growth factor (EGF) receptors in their membranes<sup>12</sup>. Studies

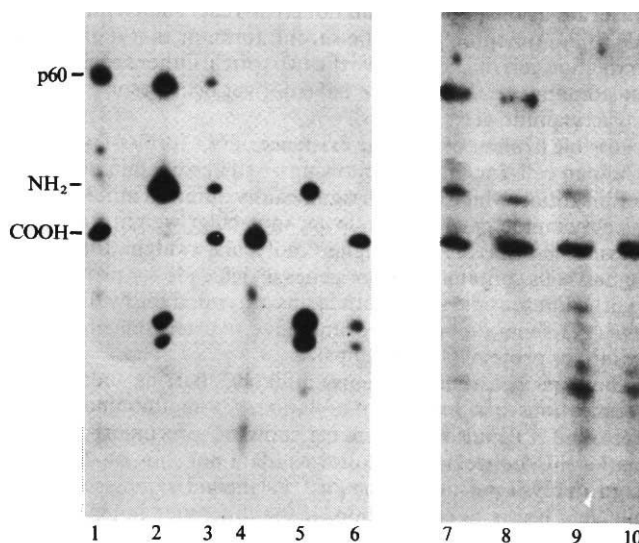


were initiated in our laboratory to compare the mechanism of growth stimulation by EGF and by viral transformation. During this work, we found that pp60<sup>c-src</sup> immunoprecipitated from <sup>35</sup>S-methionine-labelled A431 cells could be resolved into two bands by electrophoresis in 10% polyacrylamide gels (Fig. 1, lane 1). Both forms are phosphorylated (lane 3) and their apparent molecular weights are 60,000 and 59,000. Normal human fibroblasts also contain two forms of pp60<sup>c-src</sup>, both of which are phosphorylated (lane 5); about one-third of the pp60<sup>c-src</sup> is in the higher molecular weight form and two-thirds in the smaller form. Thus all human cells may contain two forms of pp60<sup>c-src</sup>. Our findings confirm recent reports that A431 cells contain a pp60<sup>c-src</sup>-like kinase activity, as assayed by the phosphorylation of tumour-bearing rabbit (TBR) immunoglobulin heavy chain in immune precipitates from A431 cells<sup>13,14</sup>.

To confirm that the two proteins immunoprecipitated from A431 cells were actually pp60<sup>c-src</sup>, we compared limited digests of the two forms with authentic pp60<sup>c-src</sup> immunoprecipitated from chick embryo fibroblasts. Partial digestion of pp60<sup>c-src</sup> or pp60<sup>src</sup> with V-8 protease gives a distinctive pattern; a single cleavage gives a 34,000-*M<sub>r</sub>* amino-terminal fragment phosphorylated at serine and a 26,000-*M<sub>r</sub>* carboxy-terminal fragment phosphorylated at tyrosine<sup>10,15</sup>. Figure 2 shows that the



**Fig. 1** A431 cells, obtained from G. Todaro, were maintained on 100-mm plastic Petri dishes (Lux) in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% calf serum (Flow). Normal human foreskin fibroblasts (obtained from T. Puck) were maintained in DMEM containing 15% fetal calf serum (Flow). For <sup>32</sup>P labelling, confluent cultures were starved for 0.5 h in phosphate-free medium lacking serum. Cells were then labelled for 2–3 h in phosphate-free medium containing 1% dialysed calf serum and 1 mCi ml<sup>-1</sup> of <sup>32</sup>P-orthophosphate (ICN). <sup>35</sup>S-methionine labelling involved first starving cells for 0.5 h in methionine-free medium containing 1% calf serum, then labelling for 2–5 h in methionine-free medium containing 1% calf serum and 50–300 μCi ml<sup>-1</sup> <sup>35</sup>S-methionine (Amersham). Radiolabelled cells were washed in 0.15 M NaCl, 0.05 M Tris, pH 7.2, and 0.001 M disodium EDTA, lysed in RIPA (10 mM Tris pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) and clarified by centrifugation for 30 min at 100,000g at 4 °C. Normal rabbit serum or TBR serum<sup>2</sup> that cross-reacted with pp60<sup>c-src</sup> was added to each lysate and immune complexes collected using protein A-containing *Staphylococcus aureus*<sup>18</sup>. The bacteria were washed once in 1 M NaCl, 0.01 M Tris pH 7.2, 0.1% NP40 and twice in RIPA in which the SDS was replaced with 1 M urea. Samples were resuspended in 0.07 M Tris pH 6.8, 11% glycerol, 0.003% bromophenol blue, 3% SDS, 5% β-mercaptoethanol and heated to 95 °C for 1 min. Proteins were separated on a 10% polyacrylamide gel using the buffer system of Laemmli<sup>19</sup>. Acrylamide (Eastman) recrystallized from chloroform and *N,N'*-methylene bisacrylamide (Eastman) recrystallized from acetone were used to achieve good resolution. Gels were fixed in 10% acetic acid–5% methanol and dried on Whatman 3 MM paper before exposure to Dupont Cronex X-ray film with a Dupont lightning-plus intensifying screen. <sup>35</sup>S-methionine-labelled A431 cells, immunoprecipitated with TBR serum (lane 1) and normal rabbit serum (lane 2), <sup>32</sup>P-labelled A-431 (lanes 3 and 4) and normal human fibroblast (lanes 5 and 6) cells, immunoprecipitated with TBR (lanes 3 and 5) or normal (lanes 4 and 6) serum. The position of p60 is indicated.



**Fig. 2** <sup>32</sup>P- and <sup>35</sup>S-methionine-labelled A431 cells and <sup>32</sup>P-labelled chick embryo fibroblasts were immunoprecipitated with TBR serum and the pp60<sup>c-src</sup> bands resolved on preparative 10% polyacrylamide gels. The unfixed, wet gels were exposed to film. The two forms of A-431 pp60<sup>c-src</sup> were rerun on a second preparative gel before use. Each band was excised and digested by *S. aureus* V-8 protease as described by Cleveland *et al.*<sup>20</sup>. <sup>32</sup>P-labelled A431 pp60<sup>c-src</sup> 60,000-*M<sub>r</sub>* form (lanes 1 and 4), A431 pp60<sup>c-src</sup> 59,000-*M<sub>r</sub>* form (lanes 2 and 5) and avian pp60<sup>c-src</sup> (lanes 3 and 6), as well as <sup>35</sup>S-methionine-labelled 60,000-*M<sub>r</sub>* (lanes 7 and 9) and 59,000-*M<sub>r</sub>* (lanes 8 and 10) forms, were digested with either 0.005 μg (lanes 1–3, 7, 8) or 0.05 μg (lanes 4–6, 9, 10) of V-8 protease. The positions of p60 and the characteristic amino- and carboxy-terminal fragments are shown.

two forms of pp60<sup>c-src</sup> from A431 cells are differently labelled by <sup>32</sup>P. The 60,000-*M<sub>r</sub>* form is phosphorylated primarily on the carboxy terminus, whereas the 59,000-*M<sub>r</sub>* form is phosphorylated almost entirely on the amino terminus. The digestion of <sup>35</sup>S-methionine-labelled material shows that each form has an amino- and carboxyl-terminal fragment, as indicated by co-migration with fragments produced by V-8 digestion of avian pp60<sup>c-src</sup>. The results suggest that the faster mobility of the 59,000-*M<sub>r</sub>* form is caused by an alteration at the amino-terminal end of the molecule. <sup>32</sup>P-labelled forms from human fibroblasts produced similar results (data not shown).

Phosphoamino acid analysis of the A431 pp60<sup>c-src</sup> forms confirmed the V-8 digestion results. Both forms were re-electrophoresed on a second preparative polyacrylamide gel to ensure that neither was contaminated by the other form. Figure 3 shows that the 60,000-*M<sub>r</sub>* form contains about 70% phosphotyrosine and 30% phosphoserine, and the 59,000-*M<sub>r</sub>* form 99% phosphoserine and only 1% phosphotyrosine. Thus, according to both phosphoamino acid analysis and limited V-8 digestion, each form is phosphorylated predominantly at either tyrosine (60,000-*M<sub>r</sub>*) or serine (59,000-*M<sub>r</sub>*) residues. We have been unable to purify away the small amount of phosphoserine found in the 60,000-*M<sub>r</sub>* form or the phosphotyrosine found in the 59,000-*M<sub>r</sub>* form.

To compare the A431 pp60<sup>c-src</sup> forms further, <sup>35</sup>S-methionine-labelled material was isolated by preparative gel electrophoresis. Complete chymotryptic digests were prepared and separated by two dimensional chromatography/electrophoresis on thin layer cellulose sheets (Fig. 4). As shown in the mixture (Fig. 4c) and in the schematic diagram (Fig. 4d), the two forms have several peptides in common. However, there are significant differences, some of which can be accounted for by a small shift in the position of a given spot, possibly due to the differential phosphorylation observed. The positions of major peptides are similar to those found for pp60<sup>src</sup> from virus-infected chicken cells<sup>4</sup>.

Several laboratories have found only one form of pp60<sup>c-src</sup> in human cell lines<sup>8,16,17</sup>, which suggests that not all human cell lines contain two forms. Alternatively, it is possible that their

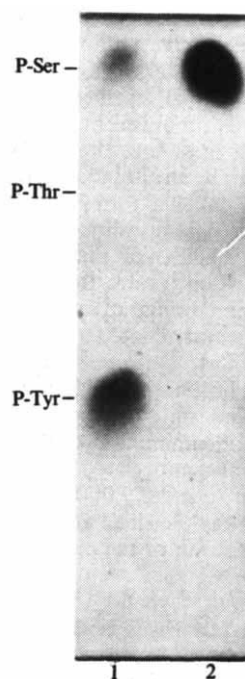
antiserum against pp60<sup>c-src</sup> did not cross-react sufficiently to be able to immunoprecipitate the second form, or that their polyacrylamide gels did not resolve the two forms. Other species may also contain two forms which have not yet been resolved using polyacrylamide gels.

Possible explanations of the existence of two forms of pp60<sup>c-src</sup> in human cells include the following. (1) They contain different modifications, which result in significantly different mobilities in polyacrylamide gels. (2) The lower molecular weight form is a cleavage product of the higher molecular weight form. (3) Human cells contain two *c-src* genes which code for proteins of slightly different sizes. (4) Both forms are coded for by the same gene, yet separate splicing events give two messenger RNAs coding for proteins of different sizes.

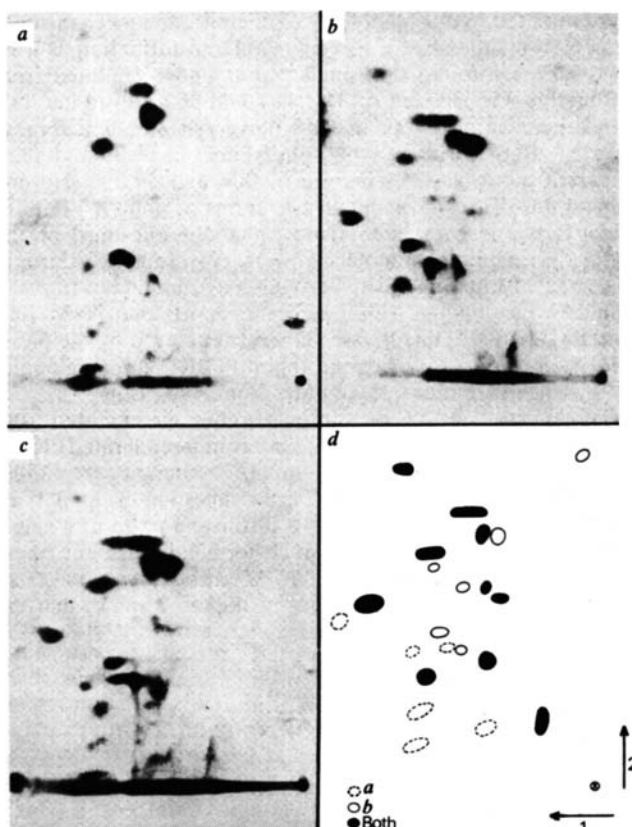
The first possibility seems unlikely, barring additional modifications besides phosphorylation. <sup>35</sup>S-methionine pulse-chase and <sup>32</sup>P pulse experiments show no precursor-product relationship between the two forms (data not shown). There is no proteolysis on incubation of <sup>32</sup>P-labelled samples at 0 °C following lysis and clarification. If the difference in mobility is due to a simple modification of the same primary amino acid sequence, then the change must be rapid and fairly stable. Experiments are in progress to examine the last two alternatives.

Even if the two forms have the same primary sequence, the fact that the two forms are phosphorylated differently is interesting. The sites of phosphorylation could alter the activity of the protein, or perhaps its location or environment. A431 membrane preparations have the same ratio of the two forms as whole cell lysates (data not shown). Experiments are in progress to separate the two forms and compare their enzymatic activities.

Thus the two human cell lines we have examined both contain two forms of pp60<sup>c-src</sup>. The two forms are phosphorylated differently, the 60,000-*M<sub>r</sub>* form containing primarily phos-



**Fig. 3** Radiolabelled proteins from <sup>32</sup>P-labelled A431 cells were prepared as described in Fig. 2 legend. The two forms of pp60<sup>c-src</sup> were eluted and precipitated using 100 µg of bovine serum albumin (Miles) as carrier and 20% trichloroacetic acid (TCA). The proteins were hydrolysed in constant boiling HCl (Pierce) for 3–4 h at 100 °C, and then lyophilysed. Each was resuspended in 3 µl of water containing authentic, unlabelled phosphoserine, phosphotyrosine and phosphothreonine and spotted on Whatman 3 MM paper. The phosphoamino acids were separated by high-voltage paper electrophoresis at pH 3.5 (pyridine/acetic acid/water, 1:10:189) for 1 h at 4,000 V. The paper was stained with ninhydrin and exposed to film with an intensifying screen. Lane 1, A431 pp60<sup>c-src</sup> 60,000-*M<sub>r</sub>* form; lane 2, A431 pp60<sup>c-src</sup> 59,000-*M<sub>r</sub>* form. Positions of ninhydrin-stained phosphoamino acid standards are shown.



**Fig. 4** Each form of A431 pp60<sup>c-src</sup> labelled with <sup>35</sup>S-methionine was prepared as described in Fig. 2 legend. The TCA-precipitated proteins were oxidized in performic acid for 1 h at 0 °C and lyophilysed twice from 1 ml of water. Proteins were then resuspended in 0.05 M ammonium bicarbonate, pH 8.5, and digested with 4 µg of chymotrypsin (Millipore) for 1 h at 37 °C. An additional 4 µg of chymotrypsin were added and digestion continued for 3 h at 37 °C. The peptides were lyophilysed, redissolved in 0.1 M ammonium hydroxide and applied to a 20 × 40 cm plastic-backed cellulose sheet (Polygram Cel 300, Macherey-Nagel). The peptides were separated by ascending chromatography (sec-butanol/*n*-propanol/isoamyl alcohol/pyridine/water, 1:1:1:3:3) and by electrophoresis at 3,000 V for 1.25 h at pH 3.5 (pyridine/acetic acid/water, 1:10:189). The sheets were exposed using Kodak X-Omat AR film at -70 °C. *a*, <sup>35</sup>S-methionine A431 pp60<sup>c-src</sup> 60,000-*M<sub>r</sub>* form; *b*, 59,000-*M<sub>r</sub>* form; *c*, mixture of both forms; *d*, schematic drawing comparing the two forms.

photyrosine, while the 59,000-*M<sub>r</sub>* form is phosphorylated mainly on serine. The V-8 and chymotryptic peptide maps clearly show that these two proteins are related. All the experiments support the conclusion that these proteins are homologous to the avian sarcoma virus transforming gene product.

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## Complex allotypes of rat $\kappa$ chains are encoded by structural alleles

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The problem of the evolution and inheritance of gene loci polymorphic for alleles with extensively differing sequences has been raised by the finding that the rat immunoglobulin  $\kappa$ -chain allotypes, RI-1a and RI-1b, which segregate as co-dominant alleles<sup>1–5</sup>, differ at 11 out of 107 amino acid positions<sup>6</sup>. It has been proposed that such 'complex allotypes' may represent the products of duplicated genes, analogous to immunoglobulin isotypes, which are subject to allelic regulation (see ref. 7 for a review). The present study was designed to determine the number and identity of the genes for  $\kappa$ -chain allotypes in the genome of inbred laboratory rats; if these allotypes represent duplicated genes it should be possible to identify the RI-1a structural gene in an inbred strain which only expresses the RI-1b allotype, or vice versa. Thus a gene corresponding to the RI-1b constant region of the  $\kappa$  light chain ( $C_\kappa$ ) was cloned from LOUVAIN (LOU) rat liver DNA, and then used as a hybridization probe to analyse liver DNA from LEW (RI-1b) and DA (RI-1a) strains of rat<sup>8</sup>. The results reported here indicate that the genome of each inbred strain contains a  $C_\kappa$  gene of only the appropriate RI-1 type. Therefore the complex RI-1 allotypes in the rat cannot be explained by duplicated genes but by an unusual evolutionary history.

The existence of multiple copies of the rat  $C_\kappa$  gene has been proposed because (1) a 10% difference in the amino acid sequence is unexpected, as the products of allelic genes usually differ by one or a very few amino acids<sup>9</sup>; (2) serological studies of inbred strains and wild rat populations of *Rattus norvegicus* have failed to identify intermediate forms or new alleles of RI-1 (ref. 10). Unlike alleles, duplicated genes could accumulate mutational changes independently of one another without the expectation of maintaining intermediate forms; and (3) the occasional expression of unexpected or 'latent' gene product has been reported, the best characterized and most relevant examples being rabbit immunoglobulin allotypes (see ref. 7 for review).

The rat  $C_\kappa$  gene was isolated by constructing a library of clones in the  $\lambda$ gtWES phage cloning vector<sup>11,12</sup>. Each phage genome contained a fragment of rat liver DNA generated by partial digestion with the restriction enzyme *EcoRI*. As there are no immunoglobulin gene rearrangements in liver DNA<sup>17</sup>, this DNA is equivalent to 'germ-line' DNA for these genes. Individual phage plaques were screened by *in situ* hybridization<sup>13</sup> using a cDNA probe corresponding to the mouse  $C_\kappa$  gene<sup>14</sup>.

The isolation, restriction map analysis and the plasmid sub-cloning of the rat  $C_\kappa$  gene have been described elsewhere<sup>15</sup>. Briefly the  $C_\kappa$ -containing clone isolated from the *EcoRI* library contained an *EcoRI* fragment bearing the  $C_\kappa$  gene which was 6.5 kilobase pairs (kbp) long. A single  $C_\kappa$  gene was identified and found to be within a *BspRI* fragment 1,170 base pairs (bp) long; the latter was then transferred to the pBR322 plasmid vector by standard cloning techniques.

Table 1 RI-1 type of  $C_\kappa$  clones

| Clone no. | Strain | RI-1 type determined by: |                       |
|-----------|--------|--------------------------|-----------------------|
|           |        | <i>DdeI</i> digest       | DNA sequence analysis |
| 1         | LOU    | b                        | ND                    |
| 2         |        | b                        | ND                    |
| 3         |        | b                        | ND                    |
| 5         |        | b                        | ND                    |
| 6         |        | b                        | b                     |
| 7         | LEW    | b                        | b                     |
| 8         |        | b                        | ND                    |
| 9         |        | b                        | b                     |
| 26        | DA     | a                        | ND                    |
| 28        |        | a                        | ND                    |
| 40        |        | a                        | a                     |

The third column shows RI-1 type as determined by digestion with the restriction endonuclease *DdeI*, for which there are allotype-associated restriction site differences within the amino acid coding region of the  $C_\kappa$  gene. DNA sequences were determined by the chemical modification method of Maxam and Gilbert<sup>18</sup>. The complete nucleotide sequence of the  $C_\kappa$  gene has been determined for clones 6 (RI-1b) and 40 (RI-1a). Partial sequences which include several allotype-associated differences have been determined for clones 7 and 9. The nucleotide sequences confirmed the allotype-associated *DdeI* site differences. ND, not determined.

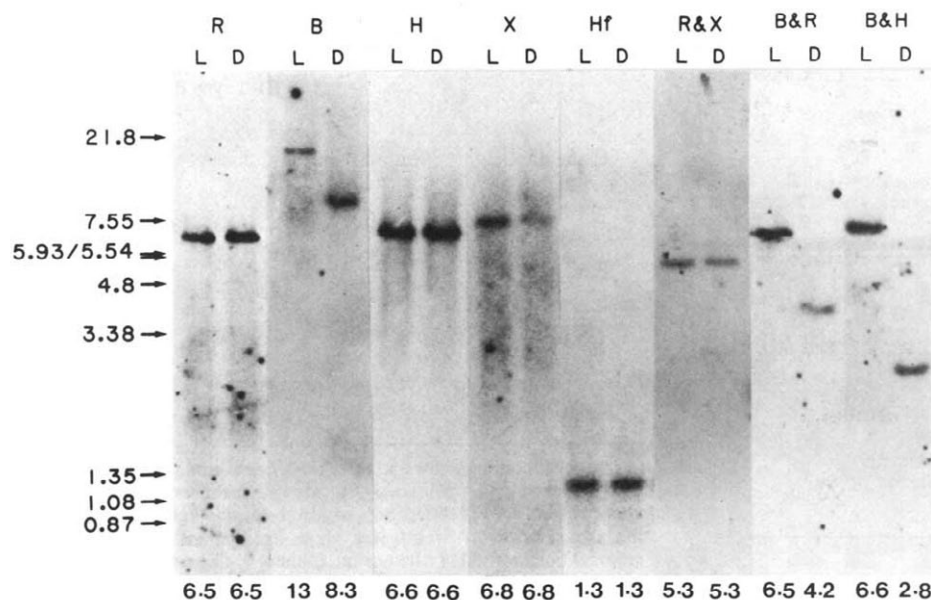
The probe used consisted of the 1,170-bp  $C_\kappa$ -containing *BspRI* fragment which was separated from the plasmid DNA after appropriate restriction enzyme digestion. The probe contains the entire  $C_\kappa$  coding region, the 3'-untranslated region, 450 bp of 5'-flanking DNA and 255 bp of 3'-flanking DNA, and it was labelled to high specific activity ( $>10^8$  c.p.m. per  $\mu$ g) by nick translation.

Initially, high molecular weight adult liver DNA<sup>16</sup> was digested with a single restriction enzyme, separated on an agarose gel, transferred to nitrocellulose paper and hybridized with the rat  $C_\kappa$  probe according to Southern<sup>8</sup>. This analysis was carried out using 8 of the 21 commercially available 'low hit' (6-base recognition site) restriction enzymes and three 'high hit' enzymes known to recognize sites within the cloned 6.5-kb *EcoRI* fragment. Four enzymes (*Clal*, *SacII*, *SalI* and *XhoI*) cut rat DNA too infrequently to give a useful range of fragment sizes (results not shown). However, six enzymes (*EcoRI*, *HincII*, *HindIII*, *HinfI*, *PvuII* and *XbaI*) each gave rise to the same single  $C_\kappa$ -containing fragment in DNA from LEW or DA rats (Fig. 1). The *EcoRI* fragment (6.5 kbp) corresponds to the fragment found in the  $C_\kappa$ -containing  $\lambda$ gtWES clone. The *HincII* (1.1 kbp) and *PvuII* (2.8 kbp) fragments have been mapped within the cloned 6.5-kb fragment. The *HinfI* (1.3 kbp), *HindIII* (6.6 kbp) and *XbaI* (6.8 kbp) fragments have not been mapped on cloned rat DNA, but correspond to fragments which may be predicted by examination of published sequences for the closely related mouse  $C_\kappa$  gene and its flanking DNA (unpublished sequences provided by E. Max).

Only one of the enzymes used for genomic blots showed an allotype-related difference, LEW having a 13-kbp *BamHI* fragment and DA an 8.3-kb fragment. This difference in *BamHI* sites has also been found in cloned DNA; the 6.5-kb fragment in the LOU clone contains no *BamHI* sites, whereas a recently cloned DA 6.5-kb fragment has a unique *BamHI* site 2.4 kb from the 5' end (data not shown).

Figure 1 shows the results of digestions using various pairs of 'low hit' enzymes. Using these data, a linear map of the restriction enzyme sites in the rat genome DNA can be constructed, covering ~13 kbp (Fig. 2).

This analysis of the rat genome has defined a region of 2.8 kbp for DA and 3.9 kbp for LEW which contains all the detectable  $C_\kappa$  sequences within a mapped region of DNA comprising ~13 kbp. Because the corresponding cloned DNA contains a single  $C_\kappa$  gene<sup>15</sup>, these results demonstrate that, unless the entire 13-kbp segment lies within a duplicated region, the rat



24 h. The blots were washed to remove non-hybridized label and exposed to Kodak X-ray film for 5–21 days using Dupont Lightning Plus intensifying screens at  $-70^{\circ}\text{C}$ . The source of the liver DNA is indicated above each lane by L (LEW) or D (DA). The restriction enzyme(s) used to digest the DNA is indicated above each pair of lanes: R, *EcoRI*; B, *BamHI*; H, *HindIII*; X, *XbaI*; Hf, *HinfI*. The size of each band (in kilobase pairs (kbp)) is indicated below each lane. These sizes were determined by graphic analysis using the *EcoRI* digestion products of phage  $\lambda$  DNA as size standards. The location and size of the standards (in kbp) are indicated in the left-hand margin.

genome of each allotype contains a single  $C_{\kappa}$  gene. Furthermore, the *BamHI* site difference between DA and LEW can be used to distinguish the two allotypes; the RI-1a (DA) genome does not contain the *BamHI* fragment found in the RI-1b (LEW) genome and vice versa (Fig. 1, lanes 3 and 4, 13 and 14, 15 and 16). If duplicated 'latent allotypes' existed in these rats, there would be a duplication of the entire 13-kbp segment with a simultaneous and independent acquisition of the *BamHI* site difference in both copies. That is, in the DA rat, both the RI-1a and RI-1b gene contexts would have to have the DA-specific *BamHI* site, while in the LEW rat, both gene contexts would lack this restriction site. This specific parallel evolution of duplicated regions seems unlikely.

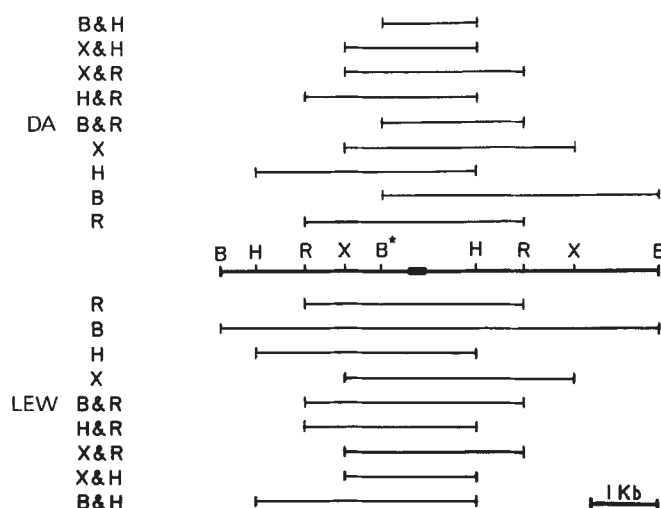
The conclusion that the rat genome contains sequences of only the appropriate allotype is also supported by analysis of independently derived clones from RI-1a and RI-1b rats. Eleven independent clones were identified using the restriction enzyme *DdeI*, which has allotype-specific sites within the coding region. In some cases, the allotype was also determined by partial DNA sequence analysis. Table 1 shows the results of the *DdeI* digests and sequence analysis of the 1,170-bp *BspRI* fragments from LOU, LEW and DA clones. The clones tested include six from the LOU (RI-1b) strain, two from the LEW (RI-1b) strain and three from the DA (RI-1a) strain. In each case the allotype of the cloned gene matched the serologically determined allotype of the corresponding rat strain. If both RI-1a and RI-1b genes existed in each strain, the likelihood of obtaining such results by chance would be extremely small ( $P < 5 \times 10^{-4}$ ).

These results demonstrate that complex allotypes, in this case differing by 10% of their amino acid sequence, can be coded for by allelic structural genes. They do not, however, exclude the possibility that the expressed RI-1 allele could be part of a large ( $>13$  kbp) duplication (that is, there may be multiple copies of the appropriate allele). Nor do they exclude the possibility that latent allotype genes exist in other species. Unlike the rabbit immunoglobulin allotypes, for example, there is no evidence for the expression of latent  $C_{\kappa}$  genes in the rat. However, these results demonstrate that it is not necessary to invoke gene duplication to account for the allelic expression of complex allotypes.

As the existence of duplicated latent RI-1 alleles in the rat is highly unlikely, two possibilities remain to explain these

complex allotypes: evolution of the two alleles by classical divergence from a common ancestral form, all intermediate forms having been eliminated by selection; and divergence of the alleles as duplicated genes, with recent gene-contraction events generating structural alleles. At present, these possibilities are indistinguishable, as they differ only in the proposed evolutionary history of the gene. However, studies on the comparative structure of other rodent  $\kappa$  chains may elucidate the precise mechanisms of their divergence.

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**Fig. 2** Genomic restriction map for LEW and DA. A linear map of restriction endonuclease cleavage sites is shown at the centre of the figure. B, *BamHI*; H, *HindIII*; R, *EcoRI*; X, *XbaI*. The relative positions of various  $C_{\kappa}$ -bearing fragments are indicated by a bar above the map for DA, and below the map for LEW. The enzyme(s) which gave rise to each fragment is indicated in the left-hand margin. The sizes were determined by graphic analysis using size standards (see Fig. 1). The position of the  $C_{\kappa}$  gene was derived from cloned *EcoRI* fragments of both LEW and DA origin (see text). The only restriction site difference between DA and LEW identified on genomic blots is indicated by an asterisk.



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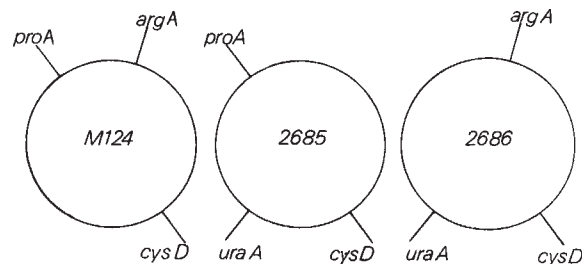


Fig. 1 Arrangement of markers in *S. coelicolor* strains M124, 2685 and 2686.

Generally, the auxotrophic requirements of the recipient strain could be transformed to prototrophy at a frequency of up to 10% while auxotrophic characteristics of the donor were expressed in ~4% of the colonies. Reciprocal experiments involving *S. clavuligerus* strains CL98 and CL198 showed that transformation to antimycin or fluorouracil resistance occurred about twice as frequently as transformation to sensitivity. If the transformation process resulted in immediate recombination and random segregation, then the two frequencies would be expected to be more equal. The discrepancies in transformation frequencies might result from the regeneration of protoplasts to form mixed colonies, or from the formation of partial diploids in which the donor DNA forms a duplication with the recipient genome. In either case, the subsequent replication and selection procedures could produce a biased sample of recombinants. To investigate this, transformed protoplasts were allowed to regenerate as a confluent culture on nonselective medium and samples of spores from this were analysed for the presence of transformant genotypes (Table 2). The results showed that, in these more rigorously nonselective conditions, the frequencies of transformation were not significantly different for both mutant and wild-type alleles when present on the donor or recipient chromosomes.

To investigate more fully the nature of the transformation process, experiments were performed in which the interaction of three genomes was studied. By using genetically well defined strains of *S. coelicolor* (Fig. 1) it was possible to investigate the recombination resulting when the DNA of one strain was used as the genetic donor and two strains served as simultaneous recipients, and when a single strain was transformed with DNA from the other two. Because of the arrangement of the marker genes carried by the three strains it was possible to identify certain classes of recombinant as having arisen from specific processes. For example, when liposome-encapsulated DNA from strain M124 was mixed with protoplasts of strains 2685 and 2686, recombinants of genotype *proA*<sup>+</sup> *argA*<sup>+</sup> *uraA*<sup>+</sup> could only have arisen from the fusion of strains 2685 and 2686, while the genotype *proA*<sup>+</sup> *argA*<sup>+</sup> *uraA*<sup>+</sup> could only be formed by the transformation of strain 2685 and *proA*<sup>+</sup> *argA*<sup>+</sup> *uraA*<sup>+</sup> by transformation of strain 2686. By measuring the preponderance of each of these diagnostic genotypes among the colonies that developed after treatment of protoplasts and liposomes with different concentrations of PEG of different molecular weight (MW), it was possible to assess simultaneously how these conditions influenced both protoplast fusion and liposome-mediated transformation. The results showed that the optimum conditions for liposome-protoplast interactions generating transformants were very similar to the requirements for protoplast fusion, that is, >40% PEG of MW 1,000 (ref. 5), rather than those for plasmid transformation (20% PEG of MW 1,000)<sup>6</sup>. This suggests that the liposome functions as an artificial protoplast and that the process of liposome-mediated transformation mimics protoplast fusion.

Table 3a shows the frequencies of all the genotypes detected among the colonies resulting from fusion of liposomes containing DNA from strain M124 with protoplasts of strains 2685 and 2686. Recombinants arising uniquely from the fusion of the protoplasts occurred at about twice the frequency of recombinants from the transformation of either protoplast.

## Liposome-mediated transformation of streptomycetes by chromosomal DNA

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Transformation is a process of unidirectional transfer of genetic information in which DNA originating from one cell is taken up by another and stably maintained<sup>1</sup>. Among the actinomycetes, probably the most interesting and important genus of bacteria, there is only one convincing description of transformation by chromosomal DNA<sup>2</sup>. It has been suggested that the absence of such transformation in streptomycetes may be due to the production of extracellular DNase<sup>3</sup>. DNA encapsulated in liposomes is resistant to the action of DNases and liposomes can be made to fuse with cells by treatment with polyethylene glycol (PEG)<sup>4</sup>. Here we describe the fusion of liposomes containing chromosomal DNA with protoplasts of streptomycetes, resulting in the formation of genetically characterized transformants.

When phospholipids are dispersed in an aqueous solution, vesicles known as liposomes form, encapsulating discrete volumes of the aqueous phase inside a bilayered lipid membrane. The addition of a solution of DNA to a film of dried phospholipid results in the formation of liposomes containing DNA which can be made to fuse with protoplasts by treatment with PEG. DNA encapsulated in liposomes is apparently released into the cytoplasm of protoplasts during fusion, as it is possible to detect changes in the genetic constitution of colonies regenerating from the fusion mixture. Table 1 shows the frequency of phenotypic transformation of *Streptomyces clavuligerus* and *Streptomyces coelicolor*. After fusion with liposomes containing DNA, protoplasts were allowed to regenerate on non-selective medium and form colonies which were then replica-plated on to diagnostic media. The numbers of colonies growing on replica plates of each selective medium were used to calculate the frequency of transformation for each marker. The results indicated that transformation of marker genes conferring resistance or sensitivity to antibiotics, auxotrophy or prototrophy could take place at high frequencies (2–10%) in both *S. clavuligerus* and *S. coelicolor*.

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**Table 1** Transformation of *S. clavuligerus* and *S. coelicolor* after PEG-induced fusion with liposomes containing DNA

| Strain | Donor DNA genotype |             |             |             |             |             | Strain | Recipient protoplast genotype |             |             |             |             |             | % Of colonies showing transformation to the indicated phenotype |                   |                   |                   |                   |                   |                        |
|--------|--------------------|-------------|-------------|-------------|-------------|-------------|--------|-------------------------------|-------------|-------------|-------------|-------------|-------------|-----------------------------------------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|------------------------|
|        | <i>hyp1</i>        | <i>ade1</i> | <i>ant1</i> | <i>trp1</i> | <i>met1</i> | <i>fur</i>  |        | <i>hyp1</i>                   | <i>ade1</i> | <i>ant</i>  | <i>trp1</i> | <i>met1</i> | <i>fur</i>  | Hyp                                                             | Ade               | Ant               | Trp               | Met               | Fur               |                        |
| N      | +                  | +           | +           | +           | +           | +           | CL198  | -                             | -           | -           | +           | +           | +           | 2.2 <sup>+</sup>                                                | 10.0 <sup>+</sup> | 6.0 <sup>+</sup>  | NT                | NT                | NT                | <i>S. clavuligerus</i> |
| CL7    | -                  | +           | +           | +           | +           | +           | CL98   | +                             | +           | +           | -           | -           | -           | 4.4 <sup>-</sup>                                                | NT                | NT                | 8.1 <sup>+</sup>  | 7.8 <sup>+</sup>  | 8.0 <sup>+</sup>  |                        |
| CL98   | +                  | +           | +           | -           | -           | -           | CL198  | -                             | -           | -           | +           | +           | +           | 3.7 <sup>+</sup>                                                | 8.8 <sup>+</sup>  | 2.2 <sup>+</sup>  | 8.1 <sup>-</sup>  | 3.1 <sup>-</sup>  | 2.4 <sup>-</sup>  |                        |
| CL198  | -                  | -           | -           | +           | +           | +           | CL98   | +                             | +           | +           | -           | -           | -           | 4.0 <sup>-</sup>                                                | 4.8 <sup>-</sup>  | 9.0 <sup>-</sup>  | 8.8 <sup>+</sup>  | 9.1 <sup>+</sup>  | 4.0 <sup>+</sup>  |                        |
| CL198  | -                  | -           | -           | +           | +           | +           | N      | +                             | +           | +           | +           | +           | +           | 4.0 <sup>-</sup>                                                | 3.9 <sup>-</sup>  | 9.9 <sup>-</sup>  | NT                | NT                | NT                |                        |
| CL198  | -                  | -           | -           | +           | +           | +           | CL198  | -                             | -           | -           | +           | +           | +           | <0.2 <sup>+</sup>                                               | <0.2 <sup>+</sup> | <0.2 <sup>+</sup> | NT                | NT                | NT                | <i>S. coelicolor</i>   |
|        | <i>proA</i>        | <i>argA</i> | <i>cysD</i> | <i>hisA</i> | <i>uraA</i> | <i>strA</i> |        | <i>proA</i>                   | <i>argA</i> | <i>cysD</i> | <i>hisA</i> | <i>uraA</i> | <i>strA</i> | Pro                                                             | Arg               | Cys               | His               | Ura               | Str               |                        |
| A3(2)  | +                  | +           | +           | +           | +           | +           | 2709   | -                             | -           | -           | -           | -           | -           | 10.1 <sup>+</sup>                                               | 10.2 <sup>+</sup> | 9.3 <sup>+</sup>  | 9.8 <sup>+</sup>  | 9.2 <sup>+</sup>  | 9.2 <sup>+</sup>  |                        |
| M124   | -                  | -           | -           | +           | +           | +           | M130   | +                             | +           | +           | -           | -           | -           | 6.1 <sup>-</sup>                                                | 5.9 <sup>-</sup>  | 2.8 <sup>-</sup>  | 9.8 <sup>+</sup>  | 9.1 <sup>+</sup>  | 8.0 <sup>+</sup>  |                        |
| M130   | +                  | +           | +           | -           | -           | -           | M124   | -                             | -           | -           | +           | +           | +           | 10.0 <sup>+</sup>                                               | 9.7 <sup>+</sup>  | 9.9 <sup>+</sup>  | 7.1 <sup>-</sup>  | 4.1 <sup>-</sup>  | 4.2 <sup>-</sup>  |                        |
| 2709   | -                  | -           | -           | -           | -           | -           | A3(2)  | +                             | +           | +           | +           | +           | +           | 4.9 <sup>-</sup>                                                | 4.5 <sup>-</sup>  | 4.7 <sup>-</sup>  | 4.7 <sup>-</sup>  | 4.9 <sup>-</sup>  | 4.8 <sup>-</sup>  |                        |
| 2709   | -                  | -           | -           | -           | -           | -           | 2709   | -                             | -           | -           | -           | -           | -           | <0.2 <sup>+</sup>                                               | <0.2 <sup>+</sup> | <0.2 <sup>+</sup> | <0.2 <sup>+</sup> | <0.2 <sup>+</sup> | <0.2 <sup>+</sup> |                        |

Extraction of DNA was performed using the procedure of Marmur<sup>10</sup> with the following modifications: (1) mycelia (2–5 g) collected after 4 days' growth in liquid complete medium (CM) were treated for 10 min with 5 ml of 10 mg<sup>-1</sup> lysozyme (Sigma) in 0.25 M Tris pH 8 and 3 ml of 20% sucrose, which resulted in a characteristic change in the appearance of the mycelium rather than lysed cells. Lysis was achieved by the addition of 3 ml of hot (90 °C) 25% SDS. (2) Diethylpyrocarbonate (0.01 ml ml<sup>-1</sup>; Sigma) was used in conjunction with EDTA to inhibit the action of DNases. Phospholipids were isolated from the chloroform–isoamyl alcohol phase of the DNA extraction procedure. 7-Dehydrocholesterol (10 µg ml<sup>-1</sup>) was added to the pooled chloroform extractions of both streptomycetes and 3 ml were evaporated to dryness under vacuum. Liposome-encapsulated DNA was prepared by adding 0.1 ml of a 10 µg ml<sup>-1</sup> DNA solution in G buffer (0.015 M NaCl, 0.0015 M trisodium citrate, 0.28 M sucrose, 0.001 M CaCl<sub>2</sub>, 0.1 M threonine, 0.1 M histidine) to the lipid film and spinning the flask (on a rotary evaporator) for 10 min. The resulting liposomes were suspended in 5 ml of P buffer<sup>11</sup> and shaken vigorously for a further 10 min, after which the concentration of visible liposome-like structures was estimated by haemocytometer counting (0.2 × 10<sup>7</sup> ml<sup>-1</sup>). Liposomes prepared in this way were very heterogeneous in size, having diameters of ~10–0.1 µm with most being ~1 µm, which is a little smaller than the average size of *Streptomyces* protoplasts. Protoplasts were generally prepared according to the method of Hopwood and Wright<sup>5</sup>. Occasionally the mycelia used were grown on cellophane disks on CM agar rather than in liquid culture. Liposomes and protoplasts were mixed in equal numbers and pelleted by centrifugation. PEG treatment was as for protoplast fusion<sup>11</sup> (50% PEG of MW 1,000 for 1 min). Suitable dilutions of the fusion mixture were spread on to regeneration medium and incubated at 25 °C. Mature colonies were replica-plated on to diagnostic media. The numbers of colonies found on each medium were used to estimate the frequency of transformation of each marker gene. In each case at least 500 colonies were tested. The mutant strains of *S. coelicolor* A3(2) used were from Professor D. A. Hopwood and were all SCP1<sup>-</sup> and SCP2<sup>-</sup>. The marker genes have been described elsewhere<sup>12</sup>. The strains of *S. clavuligerus* were isolated in this laboratory by J. Coleman; they are all derivatives of *S. clavuligerus* strain ATCC 27064 (designated by N here) obtained by mutagenesis with UV light. The gene symbols are: *hyp*, *trp*, *met*, *ade*, auxotrophic requirements for hypoxanthine, tryptophan, methionine and adenine, respectively; *ant*, *fur*, resistance to antimycin and fluorouracil. This procedure could theoretically produce liposomes each containing DNA equivalent to 10 copies of the genome. However, UV-absorbance spectra indicated that at least 50% of the DNA remained in the supernatant after centrifugation. A further 20% could be removed by treating the liposomes with DNase, which suggests that it was associated with the outside of the vesicle and thus may not have been involved in transformation. Therefore our best estimate of the DNA content of each liposome is between 3 and 5 times the size of a single genome. Control experiments were: (1) treating donor DNA with DNA exonuclease (Sigma type III, 15 units for 1 h at 37 °C, pH 5); (2) use of calf thymus DNA (Sigma) as donor; (3) mixing liposome-encapsulated DNA with protoplasts in the absence of PEG; (4) fusion of liposomes containing G buffer with protoplasts, either with or without added DNA. None of these procedures gave any evidence of transformants. In addition, it was not possible to isolate streptomycetes from the DNA solution or liposome preparations, nor any transformant-type colonies from recipient protoplasts treated with PEG in the absence of liposomes. NT, not tested.

In further experiments, transforming DNA of two different strains was carried in either the same or separate liposomes and fused with protoplasts of a third strain. When the DNAs were carried in separate liposomes (Table 3b) the frequencies of recombinants originating from a single transformation by either species of DNA were high and equal, while the genotype *proA uraA*, which could result from transformation of the protoplasts by either species of DNA, was about half as frequent. This result was surprising in that the expected frequency of this class was twice that of either unique single transformation. Thus there was possibly some selection against this class of multiple auxotroph. The number of recombinants classified as double transformants, arising from the fusion of a protoplast with at least two liposomes, was small. The genotypes diagnostic of this event require two double cross-overs between the donor DNAs and the genome of the recipient protoplast;

thus they might be expected to occur less frequently than recombinants showing only a single genetic change. However, when the same two species of DNA were encapsulated within the same liposomes and fused with protoplasts (Table 3c), the frequencies of unique single or double transformants requiring either one or two double cross-overs and the genotypes that could be formed from the interaction of either species of donor DNA were approximately equal. The generally lower frequencies of unique single transformations found in this experiment could reflect the fact that the effective concentration of each species of DNA per liposome was half. However, there was no indication that the *uraA* mutation, which was carried by both species of DNA, was donated at any greater efficiency than either *proA*<sup>+</sup> or *argA*<sup>+</sup>, which were markers unique to 2686 and 2685 respectively. In addition, the overall frequency of transformation of each individual marker in M124 was about the same in both experiments (mean 10.8 ± 2 and 9.3 ± 1).

Thus, the process of liposome-mediated transformation can produce recombination in strains of streptomycetes at sufficiently high frequency for it to be easily detectable in non-selective conditions. It seems to operate by a mechanism similar to protoplast fusion, possibly involving the coalescence of liposome and protoplast membranes and the mixing of their contents. It cannot be detected in the absence of PEG or when liposomes containing only buffer are mixed with protoplasts and added DNA before PEG treatment. As both *S. clavuligerus* and *S. coelicolor*, which are taxonomically distinct species, can be transformed in the manner described here, the method should be applicable to most, if not all, streptomycetes. We believe that this system could provide a useful addition to the well developed systems of conjugation and protoplast fusion as well as complementing the recent successes<sup>7–9</sup> in cloning streptomycete DNA on plasmid or phage vectors.

We thank K. F. Chater, Julie Coleman, D. A. Hopwood, G. Saunders and T. Savidge for encouragement and discussions.

**Table 2** Frequencies of transformation of *S. coelicolor* and *S. clavuligerus* assessed after nonselective isolation

| Donor DNA | Recipient protoplasts | % Transformation  |                   |                   |                   |                  |                  |                        |
|-----------|-----------------------|-------------------|-------------------|-------------------|-------------------|------------------|------------------|------------------------|
|           |                       | <i>proA</i>       | <i>hisA</i>       | <i>argA</i>       | <i>cysD</i>       | <i>strA</i>      | <i>uraA</i>      |                        |
| M124      | M130                  | 9.1 <sup>-</sup>  | 9.8 <sup>+</sup>  | 10.0 <sup>-</sup> | 10.0 <sup>-</sup> | 9.7 <sup>+</sup> | 9.5 <sup>+</sup> | <i>S. coelicolor</i>   |
| A3(2)     | 2709                  | 10.0 <sup>+</sup> | 10.2 <sup>+</sup> | 9.8 <sup>+</sup>  | 8.1 <sup>+</sup>  | 8.1 <sup>+</sup> | 9.3 <sup>+</sup> |                        |
| 2709      | A3(2)                 | 9.8 <sup>-</sup>  | 10.1 <sup>-</sup> | 9.5 <sup>-</sup>  | 9.3 <sup>-</sup>  | 9.7 <sup>-</sup> | 9.1 <sup>-</sup> |                        |
|           |                       | <i>trp1</i>       | <i>met1</i>       | <i>fur</i>        | <i>hyp1</i>       | <i>ade1</i>      | <i>ant</i>       | <i>S. clavuligerus</i> |
| CL98      | CL198                 | 8.8 <sup>-</sup>  | 8.1 <sup>-</sup>  | 8.0 <sup>-</sup>  | 8.5 <sup>+</sup>  | 8.9 <sup>+</sup> | 8.6 <sup>+</sup> |                        |
| CL198     | CL98                  | 9.1 <sup>+</sup>  | 8.9 <sup>+</sup>  | 8.1 <sup>+</sup>  | 9.5 <sup>-</sup>  | 9.1 <sup>-</sup> | 9.2 <sup>-</sup> |                        |

Fusion mixtures were spread at suitable dilutions to give nearly confluent lawns of growth on regeneration medium. Spore suspensions obtained from these plates were spread on to CM medium to give ~50 colonies per plate. These colonies were then replica-plated on to diagnostic media to detect the presence or absence of the various genetic markers. Genotypes of the strains are given in Table 1. The percentages shown for *S. coelicolor* were calculated from a total of 461 colonies tested, and those for *S. clavuligerus* from a total of 548.



**Table 3** Frequencies of various recombination events during three-component transformations of *S. coelicolor*

|          | <i>proA</i> | Genotype<br><i>argA</i> | <i>uraA</i> | Origin of genotype                     | No.<br>detected | % Of<br>total | No. of<br>cross-overs* |
|----------|-------------|-------------------------|-------------|----------------------------------------|-----------------|---------------|------------------------|
| <i>a</i> | —           | +                       | —           | Parental (2685)                        | 201             | 31.4          | 0                      |
|          | +           | —                       | —           | Parental (2686)                        | 271             | 42.3          | 0                      |
|          | +           | +                       | —           | Fusion of 2685 and 2686                | 65              | 10.2          | 2                      |
|          | —           | +                       | +           | Transformation of 2685                 | 31              | 4.8           | 2                      |
|          | +           | —                       | +           | Transformation of 2686                 | 28              | 4.4           | 2                      |
|          | +           | +                       | +           | Fusion and transformation              | 18              | 2.8           | 4                      |
|          | —           | —                       | —           | Fusion and/or transformation           | 13              | 2.0           | 2                      |
|          | —           | —                       | +           | Fusion and/or transformation           | 13              | 2.0           | 4                      |
| <i>b</i> | —           | —                       | +           | Parental (M124)                        | 463             | 72.3          | 0                      |
|          | —           | +                       | +           | Transformation by 2685                 | 59              | 9.2           | 2                      |
|          | +           | —                       | +           | Transformation by 2686                 | 58              | 9.1           | 2                      |
|          | +           | +                       | —           | Double transformation                  | 1               | 0.2           | 6                      |
|          | +           | +                       | +           | Double transformation                  | 5               | 0.8           | 4                      |
|          | —           | —                       | —           |                                        | 32              | 5.0           | 2                      |
|          | —           | +                       | —           | Transformation by 2686 or 2685 or both | 8               | 1.2           | 4                      |
|          | +           | —                       | —           |                                        | 14              | 2.2           | 4                      |
|          | —           | —                       | +           | Parental M124                          | 524             | 82            | 0                      |
|          | —           | +                       | +           | Transformation by 2685                 | 19              | 3.0           | 2                      |
| <i>c</i> | +           | —                       | +           | Transformation by 2686                 | 24              | 3.8           | 2                      |
|          | +           | +                       | —           | Double transformation                  | 4               | 0.6           | 6                      |
|          | +           | +                       | +           | Double transformation                  | 20              | 3.2           | 4                      |
|          | —           | —                       | —           |                                        | 13              | 2.0           | 2                      |
|          | —           | +                       | —           | Transformation by 2685 or 2686 or both | 18              | 2.8           | 4                      |
|          | +           | —                       | —           |                                        | 18              | 2.8           | 4                      |

*a*, Frequencies of recombination events detected when liposomes ( $2 \times 10^6$ ) containing DNA from strain M124 (*proA argA cysD*) were used to transform a mixture (1:1 ratio) of protoplasts ( $2 \times 10^6$ ) from strains 2685 (*proA uraA cysD*) and 2686 (*uraA argA cysD*). *b*, Frequencies when protoplasts ( $2 \times 10^6$ ) of strain M124 were simultaneously transformed with a mixture of liposomes ( $2 \times 10^6$ ) containing DNA from either strain 2685 or 2686 (1:1 ratio) prepared separately. *c*, Same as *b* except that the liposomes were prepared containing a mixture of DNA from strains 2685 and 2686. In all cases, after fusion, the protoplasts were allowed to regenerate at high density on nonselective medium. Spore suspensions obtained from the resulting lawn were spread on to plates of CM at a dilution sufficient to give ~100 colonies per plate. These colonies served as a source of inoculum to construct 10 master plates of 64 colonies, each of which was then replica-plated on to diagnostic media for genotype analysis.

\* Number of cross-overs required to produce genotype.

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## Analysis of the terminus region of the *Bacillus subtilis* chromosome

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Considerable progress has been made in the chemical characterization of the origin of replication of the bacterial chromosome in both *Escherichia coli* and *Bacillus subtilis*<sup>1–4</sup>. This region is important in the initiation and control of replication. The replication terminus, or position at which the two replication forks meet, is located opposite the origin, but its structure is unknown despite its several interesting properties which, almost certainly, reflect a significant biological role in the overall cell cycle—it seems to inhibit the movement of replication forks<sup>5,6</sup>, it is remarkably devoid of genetic information<sup>7,8</sup> and, like the origin, it shows a specific interaction with the cell surface, probably the membrane<sup>9</sup>. Recently, it was shown that the sporulation process in *B. subtilis* could be used to label the bacterial chromosome terminus radioactively<sup>10,11</sup>. Here we have investigated the number and nature of the labelled DNA fragments generated by restriction endonuclease treatment of chromosomes labelled over progressively shorter distances in the vicinity of the terminus. The results show that termination of *B. subtilis* chromosome replication is a more specific process than it has hitherto been possible to establish. Furthermore, the findings open the way to structural studies on the terminus region and to an examination by a very direct approach of the movement of replication forks within it.

Terminus labelling of the chromosome of the thymine-requiring *dna-1* strain was performed essentially as described previously<sup>10</sup>. However, to obtain sufficient amounts of radioactive DNA for analysis, the culture ( $6 \times 10^8$  cells per ml in starvation medium containing  $5 \mu\text{g ml}^{-1}$  thymine) was filtered 60 min after the initial resuspension and the cells were quickly suspended in one-fifth volume of the same medium containing  $^3\text{H}$ -thymine ( $1.5 \mu\text{g ml}^{-1}$ , 30 Ci mmol<sup>-1</sup>). On addition of 6-(*p*-hydroxyphenylazo)uracil (100  $\mu\text{M}$ ) 10 min later, the culture was diluted back fivefold with medium containing excess unlabelled thymine ( $50 \mu\text{g ml}^{-1}$ ). The spore DNA was extracted and purified in a CsCl density gradient. Cleavage with *SalI* gave the relatively simple electrophoretic pattern shown in Fig. 1 (left panel). Approximately nine well-defined radioactive bands, ranging in size from 17 to 1.2 megadaltons (Md), above a fairly high background smear, are obvious. They are not present in equimolar amounts, but, on the basis of one copy of each, their combined size is 82 Md. The *B. subtilis* chromosome is ~3,000 Md, which means that the collection of discrete fragments could represent as little as 2.7% of it. This value seems very low for a 10-min labelling period, but replication might be proceeding very slowly in the conditions used. Examination of the 10-min terminus-labelled DNA after cleavage by *Bam*HI and *SalI*+*Bam*HI revealed relatively simple patterns with approximately 12 and 20 discrete fragments, respectively. Total *B. subtilis* DNA when cleaved by *SalI* showed a very complex pattern of fragments above a smear of material spreading from a position equivalent to >30 to <1 Md.

Figure 1 (right panel) shows the *SalI* restriction patterns for spore DNA labelled for 7.5, 5.0 and 2.5 min. A gradual simplification occurs such that the 2.5-min sample shows only five fragments. Densitometer scans are shown in Fig. 2. The significant background smear, obvious in the 7.5-min case, is eventually eliminated. With respect to the discrete fragments,

the 10.2-Md fragment gradually becomes the most prominent, while those of 9.2, 6.1 and 3.4 Md disappear altogether. Although the 1.2-Md fragment remains significant on a molar basis it is difficult at this stage to say how it is changing relative to the 10.2-Md fragment. In another experiment the labelling period was reduced to 1 min and analysis of the DNA obtained confirmed the trend in Fig. 2. The 10.2-Md fragment was clearly the major component, some labelled material was obvious in the 17–15-Md area and the 7.0-Md fragment was barely detectable. The replication order of *SalI* fragments as termination is approached from the 10-min situation seems to be (13, 6.1), (9.2, 3.4), (17, 15, 7.0), (10.2, 1.2?). The 10.2-Md fragment may replicate last, but certain aspects concerning the relative amounts of various fragments could reflect some unusual features of the termination process or the structure of the terminus itself. Thus, if termination involved the simple collision of two replication forks, moving at the same rate, in the vicinity of the 10.2-Md fragment, one would expect this fragment always to be present in the labelled DNA at a higher molar ratio than any other unique fragment. However, there is less of it than of the 7.0-Md fragment in the 7.5-min sample; this could be due to the

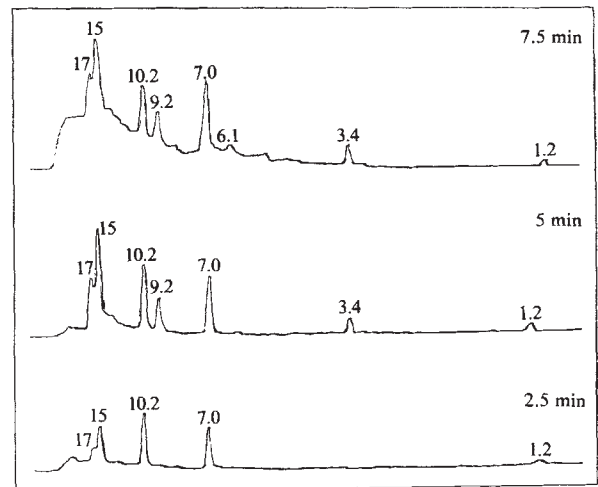


Fig. 2 Film densitometer scans of the 7.5-, 5- and 2.5-min patterns shown in Fig. 1.

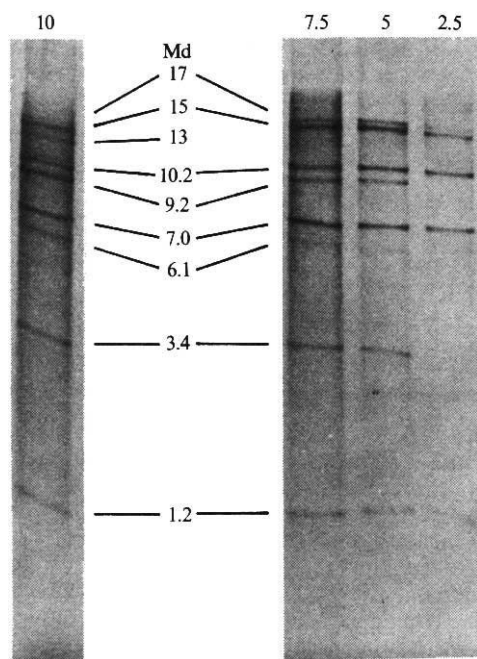


Fig. 1 Restriction endonuclease analysis of  $^3\text{H}$ -thymine terminus-labelled spore chromosomes. Spores were prepared as described in the text. For DNA isolation the labelled spores ( $\sim 7 \times 10^8$  per ml) were allowed to germinate at  $34^\circ\text{C}$  in 15 ml thymine-free casein hydrolysate medium plus 1.2% (w/v) glucose (see ref. 10). After 2 h the culture was centrifuged and the germinated spores suspended in 1 ml 10 mM Tris-HCl pH 7.5, 20% (w/v) sucrose, 0.1 M NaCl, 10 mM  $\text{NaN}_3$  and heated at  $65^\circ\text{C}$  for 10 min. 200  $\mu\text{l}$  lysozyme ( $20 \text{ mg ml}^{-1}$  in 50 mM Tris-HCl pH 7.5, 50 mM EDTA) were added at  $0^\circ\text{C}$  and the mixture incubated at  $37^\circ\text{C}$  for 60 min. Sarkosyl (1%) was added and the mixture heated at  $65^\circ\text{C}$  for 10 min and then cooled to room temperature. 500  $\mu\text{l}$  Pronase (nuclease-free,  $10 \text{ mg ml}^{-1}$  in 10 mM Tris-HCl pH 8.0, 1 mM EDTA) were added and incubation at  $37^\circ\text{C}$  continued for 60 min. The lysate was made up to 7.0 ml with 10 mM Tris-HCl pH 8.0, 1 mM EDTA. 8.75 g Optical grade CsCl were added and the mixture centrifuged in a Beckman 70 Ti rotor for 3 days at 40,000 r.p.m. The collected radioactive fractions were pooled and dialysed against SSC (0.15 M NaCl, 15 mM Na citrate pH 7.0) in the cold. DNA was precipitated with 2 vols ethanol at  $-10^\circ\text{C}$ , collected by centrifugation and dissolved in 250  $\mu\text{l}$  10 mM Tris-HCl pH 7.5, 0.2 mM EDTA. Digestion to completion with *SalI* enzyme (Boehringer-Mannheim) was performed according to standard procedures. Samples were loaded on to 0.7% agarose gels (Pharmacia) such that the total radioactivity was proportional to the labelling time used in preparing the spores. Electrophoresis was in 40 mM Tris-HCl pH 7.8, 5 mM Na acetate, 1 mM EDTA at  $1.7 \text{ V cm}^{-1}$  for 20 h (Aquebogue submarine apparatus). Molecular weight standards used were mainly  $^3\text{H}$ -thymine-labelled  $\lambda$  DNA cleaved with *EcoRI*, *BamHI* and *SalI*. Gels were prepared for fluorography as previously described<sup>1</sup>. The sample on the extreme left was obtained from 10-min labelled spores. The three on the right were from spores labelled for 7.5, 5.0 and 2.5 min as indicated.

presence of more than one copy of the latter fragment. The significance of the relatively small amount of the 17-Md fragment is also unclear. Clarification of the overall situation must await the construction of a complete map of the terminus region.

In the meantime, a direct test of the replication behaviour of the terminus fragments has been made. Spores of *dna-1*, containing 5-min terminus-labelled chromosomes, were germinated and grown out in the presence of 5-bromouracil exactly as described previously<sup>10</sup>. Replication is well under way by 75 min in these conditions but the bulk of the radioactivity does not shift into LH (light heavy) material until after the *metB* marker and at about 225 min (see ref. 10). In the present experiment, samples were taken at 150, 215 and 275 min (Fig. 3a, b and c, respectively) and the DNA fractionated in CsCl gradients. The LH and LL (light light) species were examined after digestion with *SalI* (Fig. 3). In a, where only 6% of the radioactivity was transferred to LH material (50% of the round

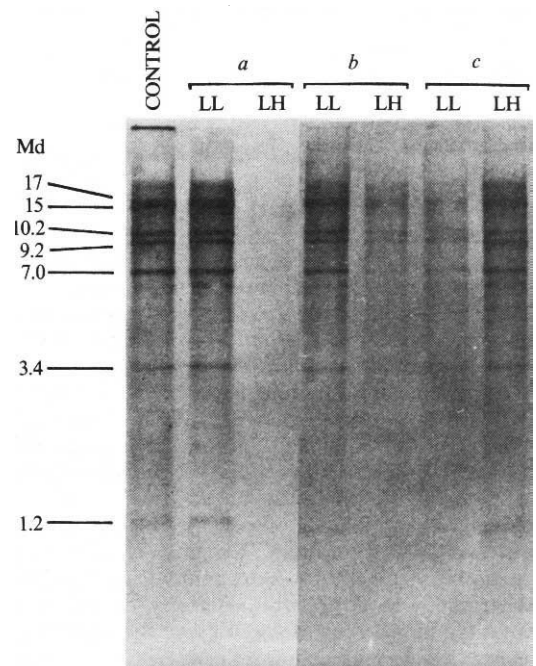


Fig. 3 *SalI* restriction patterns of the  $^3\text{H}$ -thymine DNA in pooled LL or LH species obtained after germination and outgrowth of 5-min labelled spores in the presence of 5-bromouracil (see ref. 10). In a, b and c replication was stopped at 150, 215 and 275 min, respectively. The unreplicated control is shown on the extreme left. The figure represents a composite of two electrophoretic analyses and the print has been somewhat overexposed to reveal the fainter bands.



would have been completed<sup>10</sup>), there has been no preferential replication of any of the fragments into the LH. In *b*, where 26% of the radioactivity had replicated to the LH position, the LL pattern is still unchanged and, although the LH pattern is faint, it shows no clear difference from the unreplicated material. The same situation persists in *c*, where the bulk (73%) of the radioactivity had replicated. Thus, we conclude that all *SalI* restriction fragments identified in the 5-min labelled spore chromosome terminus replicate late and as a unit when allowed to undergo a further round of replication.

The results presented here have identified the terminus region of the *B. subtilis* chromosome in terms of a collection of discrete restriction fragments. The next step required is for the order of these fragments to be defined within a map of the region, extended to include known genetic markers such as *gluA* and *citK*. The present results also show that termination occurs at a relatively specific site, at least in the strain studied here. The 2.5-min collection of fragments totals only 50 Md or <2% of the chromosome. It seems that the majority of chromosomes must terminate well within the region covered by them and probably within a region <10.2 Md or <0.4% of the chromosome.

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## Steps of mRNA translocation in protein biosynthesis

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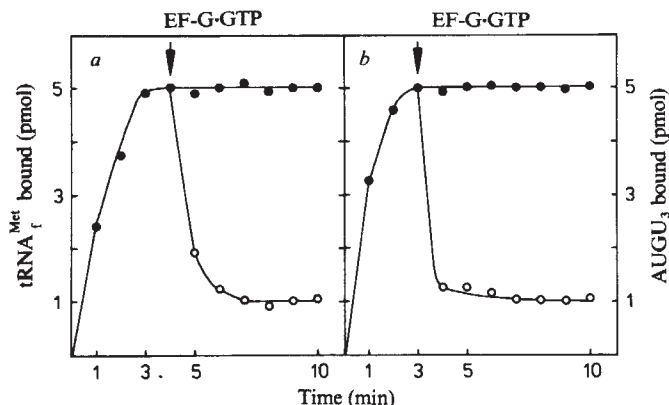
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The translocation of the messenger RNA relative to the ribosome during peptide synthesis represents an example of a mechano-chemical reaction in which the chemical bond energy of GTP is transformed into coordinated motion. Such transformations also occur during the beating of cilia and flagellae, the contraction of muscle and the migration of chromosomes in cell division. In protein synthesis the functional, geometric and energetic conditions for this transformation are well defined. For each peptide bond formed, the ribosome moves one codon along the mRNA (towards the 3' end) and one molecule of GTP is hydrolysed. Although the basic requirements of this reaction have been elucidated, the mechanism is still unresolved<sup>1,2</sup>. We demonstrate here that translocation can be analysed as a series of binding equilibria shifted by one irreversible, GTP-consuming step. The shift in the binding equilibrium is induced by the transfer of the peptidyl moiety to the (A) site-bound aminoacyl(AA)-tRNA. This results in the A site-bound tRNA having an increased affinity for the high-affinity (P) site, and a strengthened association with the mRNA. Elongation factor (EF) G · GTP catalyses removal of the deacylated tRNA, empties the P site and at the same time loosens ribosome-mRNA association. The result of these changes is that peptidyl(PP)-tRNA · mRNA is shifted from the A site to the P site, binding of AA-tRNA · EF-Tu · GTP to the vacant A site ensuring that the process is irreversible.

Figure 1a shows that addition of EF-G · GTP to a complex consisting of 70S · AUGU<sub>3</sub> · tRNA<sub>f</sub><sup>Met</sup> · Phe-tRNA reduces the amount of tRNA<sub>f</sub><sup>Met</sup> bound to the P site. EF-G is released from the ribosome after GTP hydrolysis. Thus, freeing the P site from its product, the deacylated tRNA, requires energy in the form of GTP.

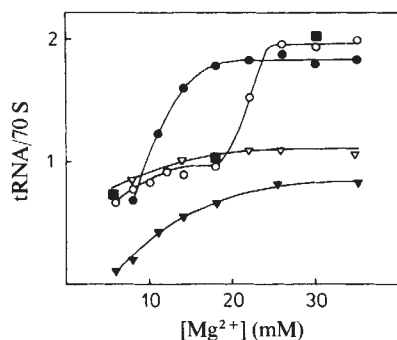
With the P site now empty, tRNA differentiates strongly between binding to the A and P sites, the 'choice' depending on the nature of their 3' substituent. Experimental support for this conclusion was obtained from the binding constants of Phe-tRNA · EF-Tu · GTP, AcPhe-tRNA and (Phe)<sub>2</sub>-tRNA to either the A or P site, which were obtained using velocity sedimentation in the analytical ultracentrifuge<sup>3</sup>. The concentrations of the oligonucleotide and macromolecular compounds were kept constant and the binding constants were shifted by varying the Mg<sup>2+</sup> concentration in a range accessible to experimental analysis (Fig. 2). Figure 2 shows that AcPhe-tRNA or (Phe)<sub>2</sub>-tRNA can discriminate between the A and P site, whereas tRNA<sup>Phe</sup> molecules bind to U<sub>13</sub>-programmed ribosomes with no obvious preference for either ribosomal site. The finding that more than 80% of the acetylphenylalanine group is transferable to puromycin in conditions of first site saturation indicates that it is the P site which is preferably occupied at 6–18 mM Mg<sup>2+</sup>.

To determine the affinity of the AcPhe-tRNA for the A site, the P site within the 70S · AUGU<sub>3</sub> complex was blocked by tRNA<sub>f</sub><sup>Met</sup> (because the initiator tRNA has a high affinity for the P site<sup>4</sup>). The ternary complex Phe-tRNA · EF-Tu · GMPPCP (guanosine 5'-(β,γ-methylene triphosphate) discriminates absolutely between the A and P sites—the Phe-tRNA binds only



**Fig. 1** *a, b*, Release of tRNA<sub>f</sub><sup>Met</sup> and AUGU<sub>3</sub> from a 70S · AUGU<sub>3</sub> · tRNA<sub>f</sub><sup>Met</sup> · Phe-tRNA complex by addition of EF-G and GTP. 70S ribosomes ('tight couples') were isolated from *Escherichia coli* MRE 600 by zonal centrifugation<sup>11</sup>. They were completely dependent on added EF-G when tested for poly(Phe) synthesis in the presence of poly(U), EF-Tu and an appropriate polymerisation mixture<sup>12</sup>. Elongation factors G and Tu were isolated from a 10<sup>5</sup>g supernatant of a ribosome preparation<sup>13</sup>. tRNA<sub>f</sub><sup>Met</sup> (1.5 nmol per A<sub>260</sub> unit) and tRNA<sup>Phe</sup> (1.3 nmol per A<sub>260</sub> unit) were from Boehringer, Mannheim. <sup>3</sup>H-CCA-tRNA<sub>f</sub><sup>Met</sup> (1,450 Ci mol<sup>-1</sup>) was a gift of Dr H. Sternbach, Max-Planck-Institut für experimentelle Medizin, Göttingen<sup>14</sup>. Aminoacylation of tRNA<sup>Phe</sup> was better than 90% (ref. 15). Oligonucleotides were synthesized with primer-dependent polynucleotide phosphorylase from *Thermus thermophilus*<sup>2</sup>. Nucleoside composition was verified with the nucleoside analyser<sup>16</sup>. Tritium-labelled oligonucleotides were prepared accordingly by using uniformly labelled <sup>3</sup>H-uridine 5'-diphosphate (100 Ci mol<sup>-1</sup>). The rate of complex formation was followed by nitrocellulose filtration using 10 pmol of 70S ribosomes and standard buffer (50 mM Tris-HCl pH 7.5, 150 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub> and 10 mM mercaptoethanol). In separate experiments ~1 μg EF-G and 0.1 μmol GTP were added after 4 min of incubation at 37 °C. In *a*, <sup>3</sup>H-tRNA<sub>f</sub><sup>Met</sup> was used as the only labelled component, in *b* it was <sup>3</sup>H-AUGU<sub>3</sub> (300 Ci mol<sup>-1</sup>). With the replacement of tRNA<sub>f</sub><sup>Met</sup> by EF-G · GTP the oligonucleotide is released simultaneously because of the low stability of the 70S · AUGU<sub>3</sub> · Phe-tRNA complex (adsorption to nitrocellulose filters). ●, Label in complex without EF-G · GTP addition; ○, label in complex after EF-G · GTP addition.

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**Fig. 2** Binding of tRNA to 70S ribosomes as studied by velocity sedimentation in the analytical ultracentrifuge. The cells were filled homogeneously with a solution containing 2  $\mu$ M tRNA, 70  $\mu$ M oligonucleotides and either 0.4  $\mu$ M ribosomes or no ribosomes. Standard buffer (see Fig. 1 legend) with varying  $Mg^{2+}$  concentration was used. The sedimentation profiles were scanned at 285 nm; temperature was 10 °C. The ribosomes and ribosome · tRNA complexes were spun down at a rotor speed of 20,000 r.p.m. for 20 min. A clear boundary of the unbound tRNA was formed after accelerating the rotor to 52,000 r.p.m. The plateau heights of tRNA boundaries were compared in the presence and absence of ribosomes, and the difference yielded the extent of tRNA bound to the ribosomes (for details of the method see ref. 3).  $tRNA^{Phe}$  (●), AcPhe-tRNA (○),  $(Phe)_2$ -tRNA (■) and Phe-tRNA · EF-Tu · GMPPCP (▽) were bound to oligouridylate-programmed ribosomes.  $(Phe)_2$ -tRNA was prepared chemically according to ref. 17. For the binding of AcPhe-tRNA to the A site (▼), the P site of AUGU<sub>3</sub>-programmed ribosomes was first occupied by one molecule  $tRNA^{Met}$ ; the increase in the extent of binding after addition of AcPhe-tRNA yielded the binding of AcPhe-tRNA to the A site; concentrations of  $tRNA^{Met}$  and AcPhe-tRNA were both 1  $\mu$ M.

to the A site. The binding constants listed in Table 1 demonstrate that AcPhe-tRNA has a 20-fold higher affinity for the P site than for the A site in our experimental conditions. Thus, the PP-tRNA should move from the A to the P site, as soon as the P site is freed from the deacylated tRNA by the action of EF-G · GTP. However, this experiment does not show how PP-tRNA carries along the mRNA in the form of a PP-tRNA · mRNA complex<sup>5,6</sup>.

Translocation requires the loosening of the ribosome · mRNA association and at the same time the tightening of the PP-tRNA · mRNA complex. The influence of the tRNA located in the P site on the stability of the association between mRNA and 70S ribosomes was determined quantitatively by the apparent binding constants of oligonucleotides to the ribosome<sup>7,8</sup>. The data in Table 2 show that the presence of two tRNAs increases the binding constants of the mRNA to the ribosome by a factor of more than 300. With only one tRNA present the affinity is increased 6–70-fold depending on the type of tRNA. The results shown in Fig. 1b support this finding.

**Table 1** Apparent binding constants of PP-tRNA to the A and P sites of programmed 70S ribosomes

| Complex                                             | $K_{ass}$ ( $M^{-1}$ ) | Stoichiometry | Binding site |
|-----------------------------------------------------|------------------------|---------------|--------------|
| 70S · U <sub>8</sub> · $tRNA^{Phe}$                 | —                      | 2             | A, P         |
| 70S · U <sub>8</sub> · AcPhe-tRNA <sup>Phe</sup>    | $3.5 \times 10^6$      | 1             | P            |
| 70S · U <sub>8</sub> · Phe-tRNA · EF-Tu · GMPPCP    | $6.6 \times 10^6$      | 1             | A            |
| 70S · AUGU <sub>3</sub> · $tRNA^{Met}$ · AcPhe-tRNA | $1.8 \times 10^5$      | 1             | A            |

Oligouridylates were prepared with polynucleotide phosphorylase and separated according to chain length by DEAE-cellulose chromatography. Phe-tRNA was acetylated with acetic anhydride (AcPhe-tRNA ~90%). The association constants were calculated from the extent of binding as taken from Fig. 2 at 8 mM  $Mg^{2+}$ . Within the ternary complex Phe-tRNA · EF-Tu · GTP, GTP was replaced by the non-cleavable GMPPCP to guarantee a stoichiometric action of the elongation factor.  $K_{ass}$  refers to binding of the underlined component to the ribosome.

**Table 2** Effect of the chain length and of cognate tRNAs on the formation of the 70S · AUGU<sub>3</sub> complex

| Complex                  | tRNA present                | $K_{ass}$ ( $M^{-1}$ ) | No. of binding sites |
|--------------------------|-----------------------------|------------------------|----------------------|
| 70S · AUGU <sub>3</sub>  |                             | $6.8 \times 10^5$      | 1.3                  |
| 70S · AUGU <sub>6</sub>  |                             | $8.7 \times 10^5$      | 1.3                  |
| 70S · AUGU <sub>13</sub> |                             | $9.3 \times 10^5$      | 1.4                  |
| 70S · AUGU <sub>3</sub>  | $tRNA^{Met}$                | $4.6 \times 10^7$      | 1.0                  |
| 70S · AUGU <sub>3</sub>  | $tRNA^{Met}$                | $3.9 \times 10^6$      | 1.0                  |
| 70S · AUGU <sub>3</sub>  | $tRNA^{Phe}$                | $4.1 \times 10^6$      | 1.2                  |
| 70S · AUGU <sub>3</sub>  | $tRNA^{Met}$ , $tRNA^{Phe}$ | $2.2 \times 10^8$      | 1.1                  |
| 70S · AUGU <sub>13</sub> | $tRNA^{Met}$ , $tRNA^{Phe}$ | $2.2 \times 10^8$      | 1.1                  |

The association constants as measured by equilibrium dialysis were derived from a Scatchard plot for 10 concentrations each. The reaction mixture contained in 100  $\mu$ l (one dialysis chamber): 50 mM Tris-HCl pH 7.5, 150 mM NH<sub>4</sub>Cl, 20 mM MgCl<sub>2</sub>, 10 mM mercaptoethanol, 20 pmol 70S ribosomes, 150 pmol of the respective tRNA(s) and 10–500 pmol AUGU<sub>n</sub> (specific activity 300–1,100 Ci mol<sup>-1</sup>). The second dialysis chamber contained only the buffer. Equilibrium was obtained at 4 °C after 8 h. The standard error of  $K_{ass}$  was calculated to be  $\pm 10\%$ . The chain length of the oligonucleotides—hexanucleotide to hexadecanucleotide—has little effect on the association constant compared with the presence of cognate tRNAs.

**Table 3** Effect of the peptidyl moiety on the stability of the oligonucleotide · tRNA or ribosome oligonucleotide · tRNA complex

| Oligonucleotide | tRNA                      | Ribosome | $K_{ass}$ ( $M^{-1}$ ) |
|-----------------|---------------------------|----------|------------------------|
| AUG             | $tRNA^{Met}$              | —        | $4.4 \times 10^3$      |
|                 | fMet-tRNA                 | —        | $1.2 \times 10^4$      |
| AUGA            | $tRNA^{Met}$              | —        | $4.0 \times 10^4$      |
|                 | fMet-tRNA <sup>Met</sup>  | —        | $1.2 \times 10^5$      |
| UUC             | $tRNA^{Phe}$              | —        | $1.7 \times 10^3$      |
|                 | Phe-tRNA                  | —        | $1.5 \times 10^3$      |
|                 | AcPhe-tRNA <sup>Phe</sup> | —        | $1.1 \times 10^4$      |
|                 | $tRNA^{Phe}$              | +        | $6.3 \times 10^5$      |
|                 | AcPhe-tRNA <sup>Phe</sup> | +        | $9.5 \times 10^6$      |
| UUCA            | $tRNA^{Phe}$              | —        | $3.2 \times 10^4$      |
|                 | Phe-tRNA                  | —        | $3.3 \times 10^4$      |
|                 | AcPhe-tRNA <sup>Phe</sup> | —        | $1.9 \times 10^5$      |
|                 | $tRNA^{Phe}$              | +        | $2.3 \times 10^6$      |
|                 | AcPhe-tRNA <sup>Phe</sup> | +        | $1.8 \times 10^7$      |

Binding constants were determined by equilibrium dialysis as described. With Phe-tRNA the pH was lowered to 6.0 to avoid deacylation of the Phe-tRNA during the 8-h incubation period. In a separate experiment it was shown that the association constant for the oligonucleotide-tRNA complex remains constant over pH 5.5–8.0. In addition to trinucleotides, tetranucleotides were used for these studies because of their increased affinity for tRNA.

Release of  $tRNA^{Met}$  from the ribosome by EF-G · GTP simultaneously destabilizes the ribosome · mRNA complex. Both experiments demonstrate that the EF-G · GTP-dependent removal of the rRNA from the ribosomal P site results in a loosening of the ribosome-mRNA association, thus predisposing the mRNA to translocation.

The effect of the tRNA's peptidyl moiety on the stability of the codon-anticodon complex was measured by comparing the association constants of cognate oligonucleotides (Table 3) with PP-tRNA, AA-tRNA and deacylated tRNA. With both tRNA species examined, the association constants between PP-tRNA and the codon were 5–20-fold higher than those of either the AA-tRNA or the deacylated tRNA. A similar effect of the peptidyl group is found in the ternary complex, 70S · UUC · AcPhe-tRNA (ref. 9).

Thus, transfer of the peptidyl moiety from the tRNA in the P site to the AA-tRNA placed in the A site, and consequent removal of the deacylated tRNA from the P site, result in PP-tRNA in the A site, and an empty P site. This PP-tRNA, however, has a higher affinity for the P site, and the peptidyl moiety induces a structural change within the tRNA which



increases the stability of codon-anticodon interaction 5–20-fold. The mRNA is held in the ribosomal complex primarily by binding to the tRNA, and so translocation may be regarded as a shift of the PP-tRNA · mRNA from its site of low affinity to that of high affinity<sup>10</sup>. Given the relevant binding constants the shift of the PP-tRNA to the P site should occur to only 95%; however, it is driven to irreversibility by the binding of the ternary complex AA-tRNA · EF-Tu · GTP to the vacant A site.

The main function of the ribosome could be to provide the specific binding sites for the components necessary for peptide bond formation and to form a cavity which prevents dilution of the PP-tRNA and the mRNA during translocation. Mechanical movement is explained as a diffusion from a low- to a high-affinity binding site. The continuous shift in one direction is guaranteed by the consecutive irreversible GTP hydrolysis. Conformational transitions of the ribosome do not seem to be necessary for this mechanism.

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## Effect of constraints, solvent and crystal environment on protein dynamics

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Both experimental and theoretical techniques<sup>1,2</sup> for the study of the internal dynamics of globular proteins are being used to delineate the highly varied motional phenomena that can occur and to relate them to specific protein functions. The method of molecular dynamics has been shown to be a powerful theoretical tool for probing the internal motions on a subnanosecond time scale<sup>2–7</sup>. Previous calculations have, however, been restricted to a protein molecule in vacuum. To determine the effect of the environment on the dynamics, a protein in solution has been simulated with a simplified solvent model and the results compared with those obtained in vacuum and in a static crystal-line environment. We report here that the presence of solvent, which results in a time-average structure closer to the native structure than the vacuum simulation, produces small alterations in the magnitudes and some changes in the decay times of the fluctuations in the interior of the protein; for surface residues, both the magnitude and the time course of the motions are altered significantly by the solvent.

The protein used, bovine pancreatic trypsin inhibitor (PTI), consists of 454 heavy atoms. (In the calculations, the hydrogen atoms were incorporated in the heavy atoms to which they are bound.) There are also four internally hydrogen-bonded water

molecules. The empirical potential function (see refs 4, 8, 9) used to obtain the forces on the atoms consists of a sum of terms associated with bond lengths, bond angles, dihedral angles, out-of-plane displacement angles, hydrogen bonds, and non-bonded van der Waals' and electrostatic interactions.

To introduce the solvent environment, one PTI molecule was placed in a box with 2,647 solvent atoms and periodic boundary conditions were applied to the system<sup>10</sup>. As the size of the box was such that there were at least four shells of solvent atoms between the protein under study and any image protein atoms, no interactions between the protein and its images were included; the system thus simulated a dilute solution. The solvent was made up of spherical van der Waals' atoms with mass, density and Lennard Jones parameters corresponding to those of a standard water model<sup>10</sup>; interactions between the solvent and protein atoms were obtained from combination rules<sup>4,8,9</sup>. Although the solvent particles do not have the directional properties of water molecules because of the absence of hydrogen-bonding interactions, they reproduce certain important solvent properties, including the generalized attractive interaction with the protein atoms, and the expected excluded volume, brownian random force and frictional damping effects.

The classical equations of motion for the protein and solvent atoms were integrated to obtain the positions and velocities as a function of time<sup>4,10</sup>. To simulate the behaviour of the native protein at equilibrium at a given temperature, the integration was started with the protein having a geometry derived from the X-ray structure<sup>11</sup> by energy minimization and the equations of motion were solved for an equilibration period before the part of the trajectory used for analysis. The motions of all the protein and solvent atoms were included explicitly in the molecular dynamics simulation, which consisted of 8 ps of equilibration at 300 K and a 25-ps run for analysis. The vacuum simulation with which the solvent run was compared used an isolated protein molecule that was equilibrated for 15 ps at 300K; the analysis part of the trajectory covered 25 ps. The vacuum and solvent runs were also compared with a simulation of one PTI molecule in the presence of the fixed force field originating from the neighbouring protein atoms in a PTI protein crystal<sup>11</sup>, but without solvent atoms; the equilibration period at 300K was 15 ps and the analysis time was 25 ps.

To reduce the cost of the solvent run, fixed bond lengths were introduced into the protein model for the dynamical simulation using the SHAKE algorithm<sup>12,13</sup>; this permitted a fourfold increase in the integration step size<sup>9</sup>. Detailed comparisons of a range of equilibrium and dynamic properties of PTI with and without bond length constraints have revealed no significant differences<sup>9</sup>. The high-frequency low-amplitude bond-length

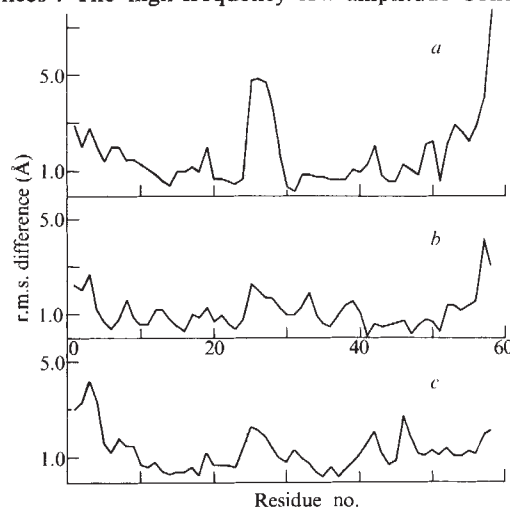
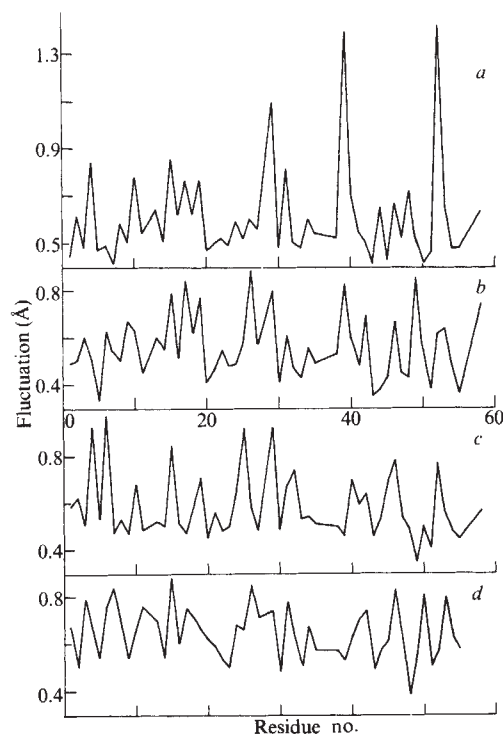


Fig. 1 Difference between the PTI calculated time-average atomic positions and the X-ray values for the C $\alpha$  atoms: a, in vacuo; b, in van der Waals' solvent; c, in static crystal environment (no solvent).

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fluctuations are only weakly coupled to the other degrees of freedom of the molecule. By contrast, a parallel simulation in which both the bond lengths and the bond angles were constrained demonstrated that the dynamics of the molecule were severely altered. The magnitude of the fluctuations of the atom positions and dihedral angles were reduced by a factor of two and dihedral angle transitions from one minimum to another were essentially eliminated. Because of the dense packing of the protein interior, the small bond-angle fluctuations ( $\pm 4^\circ$ ) have an essential role in the motions involving other degrees of freedom. Corresponding effects of bond-angle relaxation have been found in static energy minimizations<sup>14</sup>, reaction path determinations<sup>15</sup> and entropy estimates<sup>16</sup>.

An important effect of the presence of solvent is that the average dynamical structure is significantly closer to the X-ray result<sup>11</sup> than that obtained from the vacuum simulation. In the solvent run the r.m.s. deviations from the X-ray structure for the  $\alpha$ -carbons and all atoms were 1.35 and 1.94 Å, respectively,



**Fig. 2** The r.m.s. positional fluctuations for the PTI side-chain atoms averaged over each residue: *a*, in vacuum; *b*, in van der Waals' solvent; *c*, in static crystal environment (no solvent); and *d*, derived from X-ray temperature factors (see text).

compared with 2.20 and 3.02 Å in the vacuum run. Figure 1 shows the  $C^\alpha$  r.m.s. deviations from the X-ray value for the two runs as a function of residue number; in the solvent run, the  $C^\alpha$  positions were improved throughout the molecule, although the largest difference occurred for the external loop region (residues 25–29) and the ends of the chain. This effect was due in part to the external field produced by the solvent atoms, which balances the internal van der Waals' attraction and results in a more correct mean packing density and overall shape (for example, the atom number densities obtained in vacuum, in solution, and from the X-ray structure were 0.069, 0.063 and 0.060 Å<sup>-3</sup>, respectively), and in part, as for the loop region, to the solvation of exposed parts of the molecule, which counteracts their tendency to fold back on to the protein, as found in the vacuum simulation. For the crystal run, the average structure was also closer to the X-ray value, with the  $C^\alpha$  and all-atoms r.m.s. deviations being equal to 1.52 and 2.12 Å, respectively. In this case, specific interactions with the fixed crystal neighbours prevented distortions of the dynamic average structure that occurred in the vacuum simulation.

The magnitude of the r.m.s. fluctuations of the interior protein atoms was little altered by the environment; for example, for the average of the main-chain atoms (N,  $C^\alpha$ , C, O) the same magnitude (0.55 Å) was obtained in vacuum, in the presence of solvent and in the crystal. The main-chain dihedral-angle fluctuations were unaffected. Along a side chain, the solvent run values increased rather more rapidly than those obtained in vacuum. Thus for the  $C^\alpha$ ,  $C^\beta$ ,  $C^\gamma$  and  $C^\delta$  carbons, the solvated values were 0.54, 0.69, 0.89 and 1.15 Å, respectively, while those in vacuum were 0.54, 0.62, 0.74 and 0.78 Å. This is primarily a reflection of the fact that atoms further from the main chain have a greater probability of being exposed to solvent. The r.m.s. fluctuations of the side chains (atom fluctuations averaged over each side chain) are plotted in Fig. 2 as a function of residue number for the vacuum, solvent and crystal simulations, and are compared with values obtained from the experimental temperature factors (J. O. Deisenhofer, private communication) by the relation  $\langle \Delta r^2 \rangle^{1/2} = (3B/8\pi^2)^{1/2}$ ; no static disorder correction has been included in the experimental results<sup>17,18</sup> thus only the relative values are of interest. There is a good correlation between the solvated dynamics and X-ray values; the agreement is better than in the vacuum run. Furthermore, certain differences between the solvated and X-ray results can be explained by the side-chain environment: for example, Arg 39 and Glu 49, which are hydrogen-bonded to each other in the crystal<sup>11</sup> and so are confined in a steep potential well<sup>8</sup>, have very small experimental r.m.s. fluctuations. This was found for the crystal simulation but not in the solvated or vacuum run, where there are no such specific interactions.

Examination of the time course of the fluctuations shows that there were significant changes between the solvent and vacuum runs, with the crystal environment resembling the vacuum results except for the residues that interact with neighbouring protein molecules. The exterior side chains, both for the atom and dihedral angle fluctuations, showed considerable solvent damping; motion was more diffusive in the presence of solvent and relaxation times were increased. For the protein interior, the solvent effect on the time course of the motions was smaller than at the surface. However, even away from the surface there did seem to be some change (up to a factor of two) in the relaxation times of the positional fluctuations; the details of this solvent damping and its relation to the protein structure are being investigated by S. Swaminathan and ourselves.

The results of this first molecular dynamics simulation of a protein in solution support the general validity of the vacuum results for the motional behaviour in the protein interior. The primary effect of the solvent seems to be to stabilize an average structure closer to the native one. However, there are also dynamic effects; of interest is a damping of the positional fluctuation relaxation times for surface and some interior atoms. This suggests that it would be worthwhile to extend the present calculations by introducing a more complete representation of the aqueous solvent and the crystal environment.

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# MATTERS ARISING

## Neutrinos of non-zero mass in Friedmann universes

AN interesting comparison of independent methods for calculating the age of the Universe was proposed by Symbalisty *et al.*<sup>1</sup> to obtain a consistent age limit for the Universe in the range

$$13.8 \text{ Gyr} \leq T_U \leq 24 \text{ Gyr} \quad (1)$$

This has prompted us to make some further applications by using these data to impose stringent limits on the mass of light ( $\leq 1 \text{ MeV}$ ) neutrinos in Friedmann universes.

Cosmological arguments have been effectively<sup>2</sup> used to set upper limits on neutrino masses using the values of the Hubble constant,  $H_0$ , and the deceleration parameter,  $q_0$ , in a universe uniformly populated with neutrinos. We obtain here such limits with the help of the data provided by Symbalisty *et al.* without any recourse to the values of the cosmological parameters  $H_0$ ,  $q_0$ , which are in any case very uncertain.

In Friedmann models the present age  $T_U$  of the Universe is given by<sup>3</sup>

$$T_U = H_0^{-1} f(q_0), \quad (2)$$

where  $f(q_0)$  is a monotonically decreasing function of  $q_0$ , attaining its maximum value at unity when  $q_0$  approaches zero and tending to zero as  $q_0$  becomes infinitely large. Now, Einstein's equations relate the measurable quantities  $H_0$ ,  $q_0$  and the present mass density  $\rho_0$  of the Universe by the equation

$$\rho_0 = \frac{3H_0^2 q_0}{4\pi G} \quad (3)$$

This enables us to write

$$T_U = \left( \frac{3}{4\pi G \rho_0} \right)^{1/2} q_0^{1/2} f(q_0) \quad (4)$$

Let us now consider a situation at the present epoch where we can specify the mass density of the Universe and treat  $q_0$  as a parameter. It can be easily demonstrated that the function  $q_0^{1/2} f(q_0)$  increases monotonically, approaching a maximum of  $\pi/2^{3/2}$  as  $q_0$  tends to infinity. Thus, the maximum possible age,  $T_{\max}$ , for closed Friedmann models ( $q_0 > \frac{1}{2}$ ) is given by

$$T_{\max}^{\text{closed}} = \pi \left( \frac{3}{32\pi G \rho_0} \right)^{1/2} \quad (5)$$

and for open models ( $0 < q_0 < \frac{1}{2}$ ), we have

$$T_{\max}^{\text{open}} = \left( \frac{1}{6\pi G \rho_0} \right)^{1/2} \quad (6)$$

With the input of ages from Symbalisty *et al.* or from any considerations like nucleocosmochronometry, equations (5) and (6) can be used to obtain upper bounds on neutrino masses. Given the present age,  $T_U$ , the requirement  $T_U \leq T_{\max}$  provides maximum possible mass

density  $\rho_0$  for closed and open models. Let us consider neutrinos and antineutrinos of electron, muon and tau type, each having the same mass  $m$ . The present number density,  $n_{\nu}(0)$  of relic neutrinos and antineutrinos of type  $i \cong 110 \text{ cm}^{-3}$ . Then for a typical age of  $14 \times 10^3 \text{ Myr}$  the upper bounds on the neutrino mass will be 38.70 eV and 6.92 eV for closed and open models, respectively. For the age of  $20 \times 10^3 \text{ Myr}$  the same numbers will be 18.91 eV and 3.38 eV.

It is readily seen that the data on the age of the Universe place fairly tight limits on neutrino masses. The recent experimental limits<sup>4</sup> give neutrino masses of the order of a few electron volts, although the Russian experiment<sup>5</sup> reports them to be in the range 14–46 eV. Our results indicate that neutrino masses of the order of several tens of electron volts appear inconsistent with the lower limit on the age implied by nucleocosmochronometry. Again the experimental measurement of neutrino mass  $\geq 14 \text{ eV}$  is not accommodated in standard open models. Thus, if the neutrinos were to have mass of a few tens of electron volts, the data on the age suggest that neutrinos would dominate the mass of the Universe, leading to its closure<sup>6,7</sup>. Further details and some more applications of these results will be published elsewhere<sup>8</sup>.

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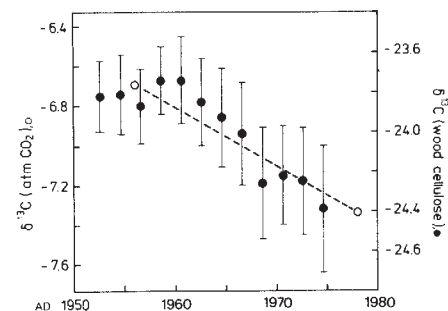
## Recent $^{13}\text{C}/^{12}\text{C}$ trends in atmospheric $\text{CO}_2$ and tree rings

FRANCEY'S<sup>1</sup>  $^{13}\text{C}/^{12}\text{C}$  record in tree rings from seven Tasmanian trees shows no systematic decrease. Such a decrease would be expected due to the dilution of the atmospheric  $\text{CO}_2$  pool by  $^{13}\text{C}/^{12}\text{C}$  deficient  $\text{CO}_2$  released by fossil fuel

combustion and forest clearing. Francey therefore questions the interpretation of  $^{13}\text{C}/^{12}\text{C}$  tree ring records in terms of global  $\text{CO}_2$  behaviour.

Measurements of more than 30 trees from the Northern Hemisphere published elsewhere<sup>2–10</sup> show, however, a systematic decrease of the  $^{13}\text{C}/^{12}\text{C}$  ratio in tree rings over the second half of the past century (measurements of eight trees over this period) and the first quarter of this century, followed by some increase of the ratio between 1940 and 1960. From about 1960 to 1975 a further decrease of the  $^{13}\text{C}/^{12}\text{C}$  ratio is observed in our more extensive tree-ring record<sup>5,6</sup> from measurements on 22 free-standing trees of different Northern Hemisphere origin (see Fig. 1). This decrease in mean  $^{13}\text{C}/^{12}\text{C}$  tree-ring data compares favourably with the  $^{13}\text{C}/^{12}\text{C}$  decrease in atmospheric  $\text{CO}_2$  between 1956 and 1978 determined directly by Keeling *et al.*<sup>11,12</sup>. Such a decrease is also found in measurements on Southern Hemisphere trees, for example one Brazilian tree determined by Rebello and Wagener<sup>13</sup>. Consequently, from a mean record calculation using all published tree-ring data I conclude that the  $^{13}\text{C}/^{12}\text{C}$  ratio in modern wood has decreased significantly<sup>14</sup>. In fact, Pearman *et al.*<sup>15,16</sup> obtained a  $^{13}\text{C}/^{12}\text{C}$  trend on three Tasmanian trees consistent with trends of some other records, which they explained as climatic implications. These three trees are included in the seven trees used by Francey<sup>1</sup> and now demonstrate the absence of any  $^{13}\text{C}/^{12}\text{C}$  trend in tree rings.

Five of the Tasmanian trees were from a rain forest. Forest trees may not reflect the change in the  $^{13}\text{C}/^{12}\text{C}$  ratio of atmospheric  $\text{CO}_2$  because of canopy effects and partial uptake of soil respiration  $\text{CO}_2$ . The  $^{13}\text{C}/^{12}\text{C}$  ratio in the latter would be



**Fig. 1**  $\delta^{13}\text{C}$  decrease in tree rings derived from about 22 trees of Northern Hemisphere origin for the 1952/53–1974/75 period (●, right scale; adjustment of numerical data from Freyer<sup>6</sup> to  $\delta^{13}\text{C} = -23.86\%$  for average AD1930–1950 wood cellulose) compared with the  $\delta^{13}\text{C}$  decrease in atmospheric  $\text{CO}_2$  observed by Keeling *et al.*<sup>11,12</sup> (○, left scale). The two scales are offset by  $-17.07\%$  (from centre of gravity coordinates) to account for isotopic fractionation between atmospheric  $\text{CO}_2$  and the average of wood cellulose in these trees. The bars of tree-ring data represent 95% confidence limits based on Student's  $t$  distribution.

delayed considerably relative to that of global atmospheric  $\text{CO}_2$  owing to the long time constant of carbon turnover in forests. The difficulty in obtaining a representative  $^{13}\text{C}/^{12}\text{C}$  record from forest trees is demonstrated by a comparison of measurements over the past 500 yr on a set of forest trees with those on a set of free-standing well-isolated trees<sup>17,18</sup>. While forest trees show large non-systematic fluctuations in the  $^{13}\text{C}/^{12}\text{C}$  ratio over the 500 yr, the  $^{13}\text{C}/^{12}\text{C}$  record for the free-standing trees shows smaller variations which can be correlated to climatic changes. Since industrialization, which began in 1850, the  $^{13}\text{C}/^{12}\text{C}$  record for the free-standing trees is dominated by a systematic decrease, which agrees with my previous record<sup>15,6</sup>.

Although the global atmospheric  $^{13}\text{C}/^{12}\text{C}$  information in tree rings is often masked by a scatter which depends on local physical as well as climatological processes<sup>14</sup>, it is, nevertheless, possible to infer past  $^{13}\text{C}/^{12}\text{C}$  ratios of atmospheric  $\text{CO}_2$ , if a larger number of free-standing trees from various parts of the world are measured, and due regard is given to parameters which influence the growth of a tree ring.

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FRANCEY REPLIES—Freyer suggests that the Tasmanian  $^{13}\text{C}/^{12}\text{C}$  tree-ring results<sup>1</sup> do not constitute grounds for doubting the interpretation of his own (mostly Northern Hemisphere) data<sup>2</sup>, in terms of global atmospheric  $\text{CO}_2$  behaviour. In my view the Tasmanian  $^{13}\text{C}/^{12}\text{C}$  records are only one of several factors which question, but do not necessarily invalidate, Freyer's interpretation.

First, I have reservations concerning the selection and combination of data used in Freyer's mean  $\Delta\delta^{13}\text{C}$  curve (Fig. 5 of ref. 2), with its large 2% decrease over the

1800–1970 period. The rejection criteria are essentially *post hoc*, and favour the removal of positive  $\delta^{13}\text{C}$  trends. Specific examples are the quite arbitrary rejection of an 1850–95 section of the  $\delta^{13}\text{C}$  record from one of our trees<sup>3</sup>, the *post hoc* decisions on duration of 'youth effects' and the arbitrary rejection of large positive  $\delta^{13}\text{C}$  excursions in the two of his trees for which full isotope data are given. The normalization and explanation of 'polluted' and 'unpolluted'  $\delta^{13}\text{C}$  records in Eifel trees<sup>4</sup> are essentially *post hoc*, and do not exclude other interpretations.

Freyer supports his present case with results from Farmer<sup>5</sup> and Tans and Mook<sup>6</sup>, amongst others. There is a massive difference (for example, if translated into an amount of  $\text{CO}_2$  of biospheric origin which must be released into the atmosphere), between the 2% decrease from 1860 to 1970 of Freyer and the <0.2% changes in the results of these authors.

The interpretation of the most recent 20 yr of data presented by Freyer must be viewed in the context of the very large discrepancies over the previous 100–150 yr. However, over this very short time scale, the lack of trend in the Tasmanian results (10 yr blocks) is not firmly established and further analysis of recent wood may well reveal the sharply increasing fossil fuel contribution to atmospheric  $\text{CO}_2$ .

Of major concern is the assumption that averaging data from a large number of trees will necessarily yield a record of atmospheric behaviour. That there are unexplained influences on  $\delta^{13}\text{C}$  in tree rings, large compared with anticipated fossil fuel effects<sup>6</sup>, is evident in Freyer's own trees<sup>2,7</sup>. If increasing industrialization, for example through rising  $\text{CO}_2$  or other atmospheric pollutant levels, influences the process of fractionation in trees, then the assumption is invalid. Previous interpretations<sup>4</sup> suggest this possibility. Recent progress in understanding the mechanism of carbon isotope fractionation during photosynthesis suggests that anthropogenic influences on fractionation will occur on both local and global scales. This will be discussed elsewhere<sup>8</sup>.

Freyer's criticism of the Tasmanian data, relating to previous whole wood results and to forest canopy effects, are specifically addressed in my original paper<sup>1</sup>. The latter effects are discussed further elsewhere<sup>8</sup>.

Although they have no bearing on the above discussion, this opportunity is taken to correct errors in Table 1<sup>1</sup>. The periods analysed for trees SC19 and BH858 should read 1743–1972 and 1885–1978 respectively.

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## 'G<sub>1</sub> rate' model of cell cycle—a realistic alternative to 'transition probability'?

RECENTLY, Castor<sup>1</sup> questioned the exponentiality of some published 'β curves' (distributions of differences between sibling cell-cycle times) and argued that his 'G<sub>1</sub> rate' model provides a better description of cell-cycle kinetics than the transition probability model<sup>2</sup>.

Regarding the form of β curves, the data examined by Castor (Fig. 1 of ref. 1) do not establish systematic deviation from an exponential. In fact, curve c (sample of 107 pairs) is well described as an exponential, as are other published examples of comparable or greater sample size<sup>2,3</sup>. Of the other data used, two sets (a and d) have too small a sample size (37 and 24 pairs, respectively) to permit any rigorous judgement. The last set (b), taken from Miyamoto *et al.*<sup>4</sup>, was obtained using a time-lapse interval of 1 h, compared with the 1.5–6-min intervals we use. This would lead to considerable uncertainty in assigning generation times, a matter of particular importance for sibling pairs having small differences in cycle times. The data also show a large positive correlation between the cycle times of mother and daughter cells ( $r = 0.648$ ) in contrast to our own published experiments where such measurements have been possible. (For example, both sets of CHO data used in ref. 2 had mother/daughter correlations that were not significantly different from zero; see also refs 5, 6.) We believe that large positive mother/daughter correlations imply either clonal or local inhomogeneities in the culture environment; at any rate, we believe that such data ought not to be used to compare cell-cycle models unless these factors have been ruled out.

We must also question the method used by Castor to estimate curvature and goodness of fit as the point chosen to assess curvature was selected arbitrarily following inspection of the data (Table 1 of ref. 1). A small sample drawn from a true exponential distribution might well display curvature about some point on a log plot, even though the chance of obtaining such curvature about any pre-selected point is quite low. Thus, it is hardly surprising that the G<sub>1</sub> rate model, with parameters chosen to reproduce the shape of a particular small random



sample, should fit the sample better than the transition probability model.

Whether the  $G_1$  rate model provides a better description of cell-cycle kinetics than the transition probability model is partly a matter of personal preference. Certainly, it can match the experimental data. However, the  $G_1$  rate model requires six arbitrary parameters, the values of which are selected by trial and error using a computer. With this degree of flexibility, it would be surprising if the model could not be made to fit. In contrast, the two-transition modification of the transition probability model<sup>2</sup> describes observed distributions of cycle times using only three parameters, the values of which are not flexible but derived uniquely from measured cell-cycle statistics. It also explains the observed correlation between sibling generation times, the lag which follows mitogenic stimulation, and the similarity of this lag to the minimum generation time of rapidly proliferating cells—unlike any other model we know of.

We also find the  $G_1$  rate model to be biologically implausible. Because the overall  $G_1$  rate for a cell is not simply the average of the rates at different 'points' in  $G_1$ , an inverse normal distribution of  $G_1$  times implies one of two things: either that each cell must maintain an invariant rate of progress through  $G_1$ , the individual rates being normally distributed, or, if  $G_1$  is considered to consist of several 'steps', that the rates of each must be perfectly correlated with the rate of the initial step, this being normally distributed in the population (that is, non-independent variance between steps). In either case, the  $G_1$  rate must effectively be predetermined at birth. Furthermore, for those cases where there is no mother/daughter correlation, the  $G_1$  rate must be reset at random for each generation. The problem then is to explain how the  $G_1$  rate can vary between cells while at the same time being predetermined at the start of  $G_1$ . We cannot think of any mechanism that could achieve this. In particular, we cannot see how such things as RNA content or protein synthetic capacity could be responsible (as suggested by Castor<sup>1</sup>) as they do not remain constant during  $G_1$ .

Regardless of mechanism, the basic contention of the  $G_1$  rate model is that variation in cycle times arises from an underlying Gaussian distribution, that is, the summation of many random variables. The 'attraction' of this is that it allows cycle variability to be ignored, or at least relegated as being unimportant. In contrast, the modified transition probability model asserts that variation is inherent to the cell cycle because two critical events occur with low probability<sup>2</sup>. Whether the latter viewpoint is eventually vindicated depends on whether the hypothetical transitions can be ascribed to real biological events, and this will not be possible by kinetics alone. However, the

parallels between the two-transition model and the biogenesis of mitotic centres may prove helpful<sup>2</sup>.

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**CASTOR REPLIES**—In considering a mechanism for the  $G_1$  rate model<sup>1</sup>, we can speculate that a cell requires a fixed quantity of a set of proteins before becoming committed to DNA synthesis, and that the rate of synthesis of these proteins is normally distributed. The random resetting in each generation of some proportion of the synthetic capacity may result from the disaggregation and reaggregation of polyribosomes at mitosis, or from different activities of nucleolar organizing regions in successive generations<sup>2,3</sup>.

Mother/daughter correlation coefficients found in several cell populations<sup>4,5</sup> are too high to be explained by experimental artefacts such as clonal heterogeneity or local inhomogeneities in the culture environment. A more likely explanation is that protein synthetic capacity is inherited to some extent from mother to daughter. Positive mother/daughter correlation can be obtained in computer simulations by combining cycle time data having different means. This procedure does not give any increase in  $\beta$  curvature. On the contrary, there is a slight decrease when data generated by the  $G_1$  rate model are combined. With regard to the 1-h frame interval of the data of Miyamoto *et al.*<sup>4</sup>, my simulations show that it yields the same  $\beta$  curvature as a frame interval of 4 min, provided the time of the former is taken as the middle of the interval. This was done for both the experimental data and the simulation of curve *b* in ref. 1.

The method used to determine the statistical significance of the curvatures gives a quantitative measure of what can otherwise be appreciated only from subjective comparisons of many successive simulations: in relatively few cases (200 of 10,000 for data set *a* and 600 of 10,000 for set *b*) does the transition probability model produce  $\beta$  curves that deviate as far from linearity as do some experimental data. Changing the midpoint of the calculation would affect these numbers

slightly, but it would not invalidate the conclusion. In contrast to the linearity of  $\beta$  curves generated by the transition probability model, the  $G_1$  rate model is consistent with both the slight downward curvature of sets *a* and *b* and the linearity (within sampling error) of sets *c* and *d*.

The 'flexibility' of the  $G_1$  rate model arises because the means, variances and measures of sibling correlation in the  $G_1$  phase and in the remainder of the cycle are potentially independent entities. Fewer parameters would be a convenience for computation, but the number of parameters does not determine whether a model realistically describes the mechanism of cycle control. A generalization such as transition probability might eventually simplify the problem, but all reasonable alternatives must be excluded before it can be accepted. It is more likely that future models will be even more complex.

The derivation of the parameters of the transition probability model from the means, variances and correlation coefficients of the cell-cycle distributions, even using several ostensibly independent calculations<sup>6,7</sup>, does not validate the model. To argue that it does is to imply that a given distribution could have been derived from only one set of conditions. In formal logic this is the fallacy of affirming the consequent<sup>8</sup>. The  $G_1$  rate model generates differences in the cycle times of siblings that conform to an exponential distribution as accurately as do populations of cells. It leads to the same means, variances and correlation coefficients as those measured for the cells, and therefore to the same mathematical relationships, without any random transition.

These considerations do not rule out a random transition as a possible explanation for cell-cycle control. The inability of the transition probability model to produce  $\beta$  curvature could be remedied by increasing its complexity by adding one or more parameters. Both models would then probably match all the kinetic data. As both models cannot possibly provide the correct explanation for cycle control, it is essential that further efforts be made to exclude one or both of them.

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## Endosperm cell number in barley

COCHRANE AND DUFFUS<sup>1</sup> have published new and interesting information on the extent of the period of cell division during barley endosperm maturation. They express concern that published observations on the endosperm of wheat<sup>2-4</sup> do not accord with their findings and offer three reasons why other workers detected a shorter period of cell division in that species. First they suggest that assays done on wheat used contaminated endosperms, but as no evidence is offered in support of this contention, one wonders to which studies this suggestion of incompetent dissection is addressed. And what of the studies which reached the conclusion that wheat endosperm divisions were completed by 16–20 days after anthesis and did not involve dissection?

Second they suggested that discrepancies between their own results (on barley) and those of others (on wheat) result from the latter having used strict chronological dating rather than their own subjective comparison system. Certainly this may contribute to differences but their extent may not be great because many workers elsewhere<sup>2-9</sup> have been in remarkably close agreement as to the time when wheat endosperm cell division ceased. It seems illogical for Cochrane and Duffus to fashion from the finding of Sandstedt<sup>6</sup> that cell divisions finished 16 days after anthesis in Nebraskan conditions, a cudgel with which to beat those who supported his conclusion from their observations on plants grown elsewhere<sup>2-5,7-9</sup>. And why do Cochrane and Duffus select Sandstedt's result as being more reliable than those which they use it to invalidate, several of which<sup>3-5</sup> were derived by similar techniques?

The third reason offered by Cochrane and Duffus is that the aleurone in barley comprises three layers, whereas in wheat it is a single layer. This seems to be unexceptionable but perhaps it is more relevant to point out that barley has at least twice as many cells in its endosperm as wheat (barley 280,000<sup>1</sup>; wheat 70,000<sup>2</sup>, 110,000<sup>3</sup>, 122,000–145,000<sup>7</sup>). It is also pertinent to observe that at 16 days after anthesis barley endosperm<sup>1</sup> comprises approximately the same number of cells as does wheat endosperm<sup>7</sup> at maturity. Divisions occurring at the same rate in both cereals would allow wheat endosperm to achieve its full complement by day 16.

It is tempting to construct a model '16-day endosperm' representing final cell number in wheat but half the final number in barley. Values extracted from Radley<sup>10</sup> (albeit from two separate experiments) suggest a 3:5 ratio between aleurone and starchy endosperm cells contributing to total cell number in mature wheat endosperm. Two further divisions of each

aleurone cell existing at this stage would produce a triple layer, as in barley, and almost double the total endosperm cell number. Such a model is consistent with Cochrane and Duffus<sup>1</sup> observations on the changes in cell number in barley endosperm 16–30 days post anthesis. Thus, there are good reasons for accepting Cochrane and Duffus' third explanation. However, they themselves seem to reject it in favour of the other two which, in their view, are compatible with the inference that they draw from Radley's<sup>10</sup> and Donovan's<sup>11</sup> publications. These do indeed provide evidence of prolonged cell divisions in wheat kernels, but Radley's experiments were not intended to reflect normal field conditions and would certainly not conform to the dating system recommended by Cochrane and Duffus. Donovan's conclusions, based on estimation of DNA at weekly intervals during kernel development, referred to whole grains. As Cochrane and Duffus observed, assays done on endosperms contaminated with other tissues can be misleading. Surely these authors are inconsistent in rejecting results obtained from what they consider to be contaminated endosperm, while espousing those obtained without any attempt having been made to remove surrounding and adjacent tissues and organs. Although the testa may be important in this context, it is likely that the embryo is far more so, for although data have not been published for wheat, it has been shown that in rye<sup>12</sup> the embryo reaches its final number of nuclei ( $1.2 \times 10^6$ ) after a period of cell division lasting twice as long as the pre-differentiation stage of the aleurone.

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COCHRANE AND DUFFUS REPLY—Evers failed to realize the significance of the work of Wardlaw<sup>1</sup> and Hoshikawa<sup>2</sup>, who have shown that temperature affects the rate of endosperm cell division. Hoshikawa<sup>2</sup> has also shown that temperature does not affect the final endosperm cell number and that cell division in the endosperm ceases earlier in plants grown at higher temperatures than in those grown at lower temperatures. Thus a caryopsis harvested 16 days after

anthesis in Nebraska<sup>3</sup> is at a more advanced stage of development than a caryopsis harvested 16 days after anthesis in England<sup>4</sup>. Evers<sup>4</sup> reported that endosperm cell division ceased at 16 days because he considered that the 'change in the appearance of the peripheral layer coincides with the conclusion of its meristematic function', whereas Sandstedt<sup>6</sup> found that divisions continued in the cells of the peripheral layer for at least 16 days from anthesis, that is, for several days after they had differentiated into aleurone cells. Simmonds and Campbell<sup>5</sup> have made similar observations in rye. Evers<sup>4</sup> photographs of transverse sections of wheat caryopses show that, between 16 and 22 days, the diameters of the cells inside the aleurone have increased while those of the aleurone have not. If the photographs are of representative sections and the aleurone remained intact, then it seems that anticlinal divisions had occurred in the aleurone cells between 16 and 22 days. Some anticlinal divisions are difficult to identify in transverse sections and so at this stage of development observations should be made on tangential sections.

The concept of a '16-day endosperm' is untenable when considered in relation to environmental variation and to the variation in endosperm cell number which exists between cultivars<sup>1,6,7</sup>. A calculation such as Evers has proposed could not take into account the increases in cell number due to anticlinal divisions in the aleurone layer.

It was not our intention to deny the significance of the differences between wheat and barley. We were, however, concerned to draw attention to the discrepancies existing in the interpretation of the work on endosperm cell division in wheat, particularly regarding the effect of environment and the contribution of the aleurone to endosperm cell number. Small increases in endosperm cell number may prove to be important, not necessarily for their contribution to yield in normal field conditions, but as an indication of the capacity of the aleurone cells to divide and hence to respond to chemical treatment aimed at increasing endosperm cell number.

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## BOOK REVIEWS

## Unravelling the energy issues

P.D. Henderson

It is not often that economists and natural scientists succeed in working together. Even in a field of inquiry such as energy, where issues and problems clearly extend across conventional subject boundaries, it is hard to achieve a meeting of minds. Hence genuinely collaborative work, as distinct from separate essays or chapters that are placed side by side, is a rather rare event. This volume, written by a team from the Energy Research Group at the Cavendish Laboratory in Cambridge, provides welcome evidence that close and effective collaboration is possible.

The intention of the authors is — to quote from the preface — “to bring students, and an informed public outside the universities, towards the frontiers of knowledge in a complex of inter-related disciplines”. Thus the book tries to bring together different aspects of energy — in particular, economic and technical aspects — in a unified and self-contained treatment, designed for a wide and non-specialized readership.

How far have the Cambridge team succeeded in their aim? As to presentation, the results are impressive. Multiple authorship, especially when different disciplines are involved, is not easy to combine with a treatment which is both readable and consistent. All too often, either individual contributions fail to make up a coherent whole, or a heavy editorial hand imposes cohesion at the price of extreme dullness. Both of these dangers are avoided here. Collective responsibility is emphasized, so much so that the names of individual authors are not attached to chapters or sections, and the book forms a unity. At the same time, the argument and supporting evidence are set out in a lucid and generally interesting way, and in the concluding chapters especially, the style is brisk and effective. All this is a considerable achievement.

When it comes to content, there is more room for doubt. The argument is set out under five headings: energy demand (two chapters with an introduction); supply factors (six extended chapters, comprising some 40 per cent of the text); the market for energy; the world energy outlook; and issues of energy policy. At each stage there is in effect a blend of information and analysis, the proportions varying according to the precise topic. The main unifying theme is that of the present world predicament. Energy data and choices are presented against the background of a complex and uncertain process of

*Energy Economics: Growth, Resources and Policies.* By Richard Eden, Michael Posner, Richard Bending, Edmund Crouch and Joe Stanislaw. Pp. 442. ISBN 0-521-23685-1. (Cambridge University Press: 1981.) £19.50, \$34.95.

transition, from dependence on low cost oil and gas to a more varied and costly regime. The chief role of energy policy is seen as being to manage this process, in such a way that disruption is avoided and general economic performance is not seriously impaired.

This makes a reasonably coherent structure, and for the most part the treatment is both informative and judicious. At the same time, the book seems to me to suffer from a serious and pervasive weakness. Partly in what it does, but still more in what it fails to do, its handling of the economic aspects is unsatisfactory.

As to what is said, I found some of the economic sections superficial, and even misleading at a number of points. This applies to the treatment of external effects in Chapter 9, and to the whole of Chapter 15, on energy and developing countries. Again, the chapters on policy, while stimulating and often very perceptive, suffer from an unduly restrictive conception of the scope of economic considerations, in which strategic and environmental aspects are treated as separate. They also contain some very dubious assertions about the role of markets in general, and of international trade in particular.

The omissions are more worrying. In particular, there is no systematic attempt to outline the economic analysis that is applicable to energy questions, and to convey to the general reader the distinctive contribution of economic modes of thinking. This contribution is important, even fundamental, despite the obvious and depressing facts that economists know little about how economic systems work and find it hard to agree with one another. In energy, as in other spheres, the standard economic theory of resource allocation is useful not only in itself, but also and perhaps mainly because of what it displaces. Most thinking about energy, even in quite exalted political, business and scientific circles, embodies a strong measure of what I call “do-it-yourself economics”, in which the main ingredients are an oversimplified conception of choice, an unreflecting acceptance of various mercantilist notions, and an excessive faith

in the predictability and controllability of events. A book on energy economics which fails to make this point, and which gives no adequate account of the standard theory and its uses, represents in my view a badly missed opportunity — an opportunity such as was brilliantly grasped, albeit in a different context, in Hitch and McKean’s *The Economics of Defense in the Nuclear Age* (1960).

The consequences of this omission extend to the treatment of policy. Actual energy policies are often strongly influenced by dubious forms of economic reasoning, and this helps to explain not only some past mistakes but also the frequent failure of those concerned to learn from experience: British nuclear power programmes provide a depressing example. Such things pass unnoticed here (and incidentally but significantly, Duncan Burn’s perceptive studies of nuclear power in Britain are not listed in the bibliography). In this respect the discussion of policy issues, though in general informed and sophisticated, has a certain unwitting innocence. The evidence of history has not been used to good effect.

*Energy Economics* is a useful and in many ways impressive book, and an encouraging instance of interdisciplinary teamwork. But unfortunately, it does not fully make good the claim implied in its title. □

P.D. Henderson is a Professor in the Department of Political Economy at University College, University of London.

## Tunnelling particles

Rudolph A. Marcus

*The Tunnel Effect in Chemistry.* By R.P. Bell. Pp.222. ISBN 0-412-21340-0. (Chapman and Hall: 1980.) £17, \$39.95.

In this small, tightly-written volume, Professor Bell describes in detail almost every aspect of the tunnelling of protons, atoms and molecules. Tunnelling, a phenomenon which is impossible in Newton’s world of classical mechanics, is associated with a particle’s wave-like behaviour, first recognized in the 1920s.

The author begins with a succinct description of the quantum mechanical origins of the tunnelling effect, considering

its optical analogue and giving its magnitude both for a number of well-known potential energy barriers to tunnelling and for the Wentzel–Kramers–Brillouin approximation to the problem. The influences of tunnelling on the parameters of an Arrhenius plot and on isotope effects in reactions are described, together with criteria for tunnelling. Professor Bell gives a clear synopsis of our understanding of these topics and discusses the relative importance of the effects of zero point energy and of tunnelling on the rate constant. Additionally, there is a brief account of the problem of curvature of the reaction path and of the means by which its effect on tunnelling may be treated.

Also included are consideration of the theories of proton transfer reactions proposed by Dogonadze and co-workers, and myself — with careful evaluation of these ideas in terms of experiment — and of the observed effects of polar and nonpolar solvents on the isotope effect. In the following detailed review of the evidence for tunnelling in chemical reactions based on abnormally large isotopic effects, Professor Bell gives some due precautionary remarks on special nontunnelling cases where abnormal effects can occur.

Most systems covered in the book involve a concerted internal motion in which the tunnelling is largely that of hydrogen atoms or protons, but examples of systems involving tunnelling of heavier

masses, such as  $\text{CH}_2\text{O}$  or  $\text{CO}$ , are given appropriately using as evidence the temperature-independent reaction rate at very low temperatures ( $\sim 4^\circ\text{K}$ ). While prominence has understandably been given to tunnelling in chemical reactions, the influence of tunnelling on molecular spectra is covered, with an interesting assortment of examples, including rotational predissociation, inversion doubling and hindered rotation.

A surprisingly large number of topics have been packed into the 200 or so pages of the book, and the instances in which some aspect of atom or proton tunnelling has not been covered are rare. Perhaps the most important omission is that there is no mention of radiationless transitions. Here, the “energy gap law” of Siebrand for triplet to ground state singlet decay rates in aromatic hydrocarbons (and for other transitions in other systems) has its origin in small Franck–Condon factors and, thereby, via semiclassical (WKB) arguments, in tunnelling of the atoms.

However, there is so much broad information made readily available in this volume that any such omissions are, indeed, to the author’s credit, almost “of measure zero”!

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be treated as autonomous organelles, and isolated and analysed without compromising the relevance of such studies to their functions in living cells. Thus at the time the symposium was proposed, the prospects for relating membrane structure to function must have seemed good.

And yet, as this book illustrates, perhaps inadvertently, in spite of considerable advances in our understanding of the nature of plasma membranes and the phenomenology of membrane-dependent processes, the molecular basis of many such phenomena remains obscure. One factor is the lack of information on the molecules actually involved in many of the functions mediated by natural membranes. But perhaps a major contributing element has been the over-emphasis on the independence of membranes from the rest of the cell. The idea that membrane components, particularly the proteins, are not fully independent has been suggested by observations such as their slow rates of diffusion, and a tendency to accumulate in specialized regions of the plasma membrane, such as microprojections, coated pits, viral assembly sites and caps. As someone once said, “Membranes may not be as rigidly fluid as once believed”! There is mounting evidence that the restricted mobility of some membrane components and their localization at particular sites is not necessarily a process of self-assembly, but that cytoplasmic structures such as microfilaments, viral matrix proteins and clathrin coats interact with the membrane components to restrain their intrinsic mobility. The implication is that to understand the molecular basis of such membrane-dependent phenomena requires an examination of both the membrane itself as well as the cytoplasmic elements involved. Particularly worrying is the fact that studies on model systems or even isolated membranes may often be inappropriate.

Thus, the apparent lack of cohesion between the two halves of this book cannot be blamed entirely on editorial judgement. Admittedly, the choice of subjects within the concept of intercellular recognition is a little arbitrary, but one has to accept that, in general, the subjects have not advanced sufficiently to generate a more coherent picture. In fact, as an assessment of the current state of membrane biology, the book has many virtues. Articles on such subjects as integral membrane proteins (Henderson), glycoproteins (Sharon) and lateral mobility (McConnell) provide balanced, up-to-date examinations of the respective fields. Since the book was produced in connection with a course of lectures, the presentations are erudite, but not esoteric, and therefore particularly valuable to the non-expert. Some articles, such as those on gap junctions (Benedetti and Dunia) and the cholinergic receptor (Popot) even come close to achieving the objectives implicit in the title.

In general, the book tells us quite a lot

## Fluid membranes and cell function

Gordon Koch

*Membranes and Intercellular Communication.* Les Houches, Session XXXIII, 1979. Edited by Roger Balian *et al.* Pp.500. ISBN 0-444-85469-X. (North-Holland: 1981.) \$122, Dfl.250.

ABOUT ten years ago the development of the concepts of fluidity, lateral diffusion and integral proteins initiated what might be called modern membrane biology. Irrespective of one’s views on the apportioning of credit, the integration of the various developments into the fluid mosaic model provided the basic framework within which the multitudinous properties of membranes have been examined. Fortunately, ten years on, after much intensive examination, the foundations of the model remain secure. One of the merits of this book is that the first part, dedicated to assessing the current status of these, succeeds in bringing one fairly well up to date with some readable articles on subjects such as lateral diffusion and integral membrane proteins.

However, the main subject of the symposium upon which the book was based was to relate what is known about the physiochemical properties of

membranes to some rather complex membrane-dependent phenomena, such as immunological recognition, hormone action and synaptic transmission. Was this an over-ambitious exercise? Not necessarily, considering some of the views on plasma membranes which have prevailed since the introduction of the fluid mosaic model. In particular, the concepts of fluidity and the mobility of membrane components have dominated our thinking about plasma membranes over the past decade, to the extent that some models for membrane processes such as the capping phenomenon, have actually assumed that all membrane components are diffusing at rates close to their theoretical maxima. If this is true, the plasma membrane must closely approximate to an ideal solution, the general implication of which is that its properties should be definable by its composition. Such a situation is, of course, extremely encouraging for those interested in determining the molecular mechanisms of membrane phenomena, since the analysis of composition is, in principle, easier than that of configuration and interaction. A practical consequence of unfettered fluidity is that membranes can



about membranes and rather little about intercellular recognition — which is not surprising, since the latter must encompass just about the whole of biology. □

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## New limbs for old

Bruce M. Carlson

*Vertebrate Limb Regeneration.* By H. Wallace. Pp.276. ISBN 0-471-27877-7. (Wiley: 1981.) £19.50, \$46.35.

ALTHOUGH one of the oldest fields in experimental biology, limb regeneration remains one of the least understood, currently being in a state similar to that of genetics just prior to the rediscovery of Mendel's laws. From numerous descriptive and experimental studies have arisen traditional dogmas (the neurotrophic theory, for example) and controversies, such as that surrounding the origin of blastema cells. The past decade has seen a burst of interest in morphogenesis and the formulation of numerous models designed to account for the observed phenomena.

In writing this book, Wallace intends to provide the reader with not only an extensive introduction to limb regeneration, but a critical review, in which he hopes "either to clarify and reinforce the traditional view or to expose deficiencies in our knowledge and interpretation of the subject". For example, he re-examines the neurotrophic concept and concludes that the evidence buttressing the threshold hypothesis (that a critical number of area of nerve fibres per cross-sectional area of limb must be exceeded for regeneration to occur) could be arranged equally well to support a stepwise relationship between nerve quantities and regeneration. He also asks whether nerves are necessary at all, and presents some arguments and unpublished data suggesting that amphibian limbs can regenerate after long-standing denervation.

Although it is unfortunate that the results of an unpublished experiment are used to deliver his *coup de grâce* to the neurotrophic theory, the author's dealing with this topic is illustrative of the way in which he examines the data upon which many of the long-held concepts in regeneration are based. While making no attempt to conceal his own personal biases, Wallace similarly examines topics, such as hormonal and irradiation effects. I admire his dissection of many issues, but feel that certain others are considered as established without being subjected to the same intellectual scalpel.

A considerable portion of the book is

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devoted to the causal analysis of morphogenesis and morphogenetic models. In this area the literature of regeneration is not noted for its objectivity. Wallace, again, makes no secret of his own personal bias, but provides a thoughtful analysis of the experimental evidence underlying most of the current models. The last chapter contains speculative material that summarizes a number of the author's own ideas on regeneration, a major one being that limb regeneration is strictly a repetition of limb development.

Whether or not one agrees with some of the arguments and conclusions, this book is valuable not only for the amount of information it contains, but also for the original thoughts and interpretations. It should meet the author's expectations of providing a stimulus for future investigation into some of the outstanding issues of limb regeneration. □

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## Academic barriers at high altitude

Peter D. Moore

*Ecology of Highlands. Monographiae Biologicae, Vol. 40.* By M.S. Mani and L.E. Giddings. Pp. 247. ISBN 90-6193-093-6. (Dr W. Junk, The Hague: 1981.) Dfl. 110, \$58.

EXTREME environments, such as the deep seas and very high altitudes, present peculiar ecological problems to the plants and animals which inhabit them. Few books have been written on the deep seas, whereas many have been written about the ecology, flora and fauna of the high mountains. But most of these go little further than to describe the unusual features of "alpine" plants and animals inhabiting those regions, often in terms of their morphology, reproductive strategies or biogeographical significance. This book attempts to go further by concentrating upon the physical environment of high altitude habitats, the effects that extreme physical conditions have upon plants and animals (including human beings) and the adaptation resulting from the evolutionary responses of these organisms.

The twin authorship reflects the two aspects of the problem. L.E. Giddings (Veracruz, Mexico) is responsible for the sections on the physics and meteorology of the habitat and M.S. Mani (Agra, India) covers the biological aspects. Such a dual approach is novel and brave, the main problem being the integration of the two sections, especially in view of the disciplinary and geographical gulf between the two authors.

Introductions are places for generalizations, which may be modified and expanded later in the text. The introduction by Mani, however, contains generalizations which have a rather inflexible ring to them. One would expect some overall definition of highland in terms of altitude. Instead, the definition concentrates upon low atmospheric pressure, cold and aridity (the latter surely being very variable). Then we are given such dogmatic statements as "The altitude at which the mean annual air temperature in the shade does not rise above 10°C is the timberline". No indication is given that

this might vary with such factors as wind. Indeed, the essentially continental (in a meteorological sense) experience of the authors in the mountains of Asia and South America is evident in the book, which will limit its value to those working in oceanic areas.

We then have eight chapters by Giddings on the physical environment of high altitudes. There are many useful and interesting sections in this part of the book, though it does suffer from a surfeit of tabulated data relating to atmospheric variables, which could have been relegated to appendices. There is also a strong emphasis upon instrumentation and upon engineering and physical features of the environment which have a very flimsy connection with its ecology. (Does the ecologist really need to know how long it will take him to boil an egg at high altitude?) Indeed, there is no systematic attempt to relate the physics to the biological problems discussed later by Mani.

Mani begins with general surveys of plant and animal life at high altitude, much of which is a condensation of his previous books on highland insects and plants. There is a strong Himalayan flavour to these chapters, but data from other areas are incorporated. The taxonomic arrangement of material is surprising and this does little to ease the transfer of authorship and emphasis, though the animal chapter does attempt to relate adaptation to physical factors.

The final chapters concentrate on human beings, especially the physiological peculiarities found in high altitude peoples.

Overall the book does not succeed in integrating the two approaches to the highland environment except in rather isolated sections of Mani's accounts of animal and human adaptation. It was an ambitious idea but, sadly, the barriers of long-independent development within the two scientific disciplines, coupled perhaps with the geographical isolation of the authors, have proved too great. □

Peter D. Moore is Senior Lecturer in the Department of Plant Sciences at King's College, University of London.



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